VALUE-ADDED FUNCTIONAL PROTEIN PRODUCTS AND ENDOGENOUS ANTIOXIDANTS FROM AQUATIC SPECIES

AKHILE COLLINS ONODENALORE

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VALUE-ADDED FUNCTIONAL PROTEIN PRODUCTS AND ENDOGENOUS ANTIOXIDANTS FROM AQUATIC SPECIES

BY

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THIS WORK IS DEDICATED

TO

MY WIFE AND CHILDREN

ABSTRACT

Protein dispersions were prepared from male and spent female capelins and shark after aqueous washings of their meats using 0.5% NaCl, 0.5% $NaHCO_3$ and water, consecutively. Extraction of pigments, odorous matter and free amino acids occurred during washings. The unacidified dispersions were highly viscous and stable to heat and centrifugal force. Over 90% of the protein in the dispersions remained in solution after heating at 70 or 100 °C. However, on addition of acetic acid, viscosity of shark protein dispersion was lowered. Total amino acid composition of the dispersion virtually remained unchanged due to washing. The protein efficiency ratio (PER) values of washed meats, calculated by an amino acid scoring methodology, were comparable to those of unwashed meats. However, over 60% of the free amino acids were extracted due to washing, thus producing a bland product.

Enzyme-assisted hydrolysis of shrimp heads and female lumpfish after roe extraction was carried out. Of the enzymes considered (Alcalase, Neutrase and Trypsin), Alcalase was the most efficient in producing protein hydrolysates with desired degree of hydrolysis (DH) and highest nitrogen recovery (NR). Response surface methodology (RSM) was employed to optimize the hydrolysis conditions using Alcalase for shrimp heads and lumpfish proteins. A polynomial regression model was used to predict the optimum conditions for the process, these were 58 °C at pH 8.1 and enzyme/substrate concentration ratio of 33.1 AU/kg crude protein for shrimp protein hydrolysis for a predicted DH of 17.8%. The optimum hydrolysis conditions

for lumpfish protein, as predicted by the regression model, were temperature, 63 °C; pH, 8.1; and enzyme/substrate concentration ratio, 28.5 AU/kg crude protein for a DH of 18.4%. Nitrogen recovery in shrimp and lumpfish protein hydrolysates prepared under optimum hydrolysis conditions were 72.2 and 79.9%, respectively. The regression coefficient (R^2) values which were >0.95 and the close values of experimental hydrolysis results to those predicted, indicated the adequacy of the models employed. The total amino acid composition of the prepared shrimp and lumpfish protein hydrolysates were similar to those of their starting materials. However, over a 14-fold increase in the content of free amino acids was noted in these protein hydrolysates. Furthermore unique functional characteristics of the hydrolysates with respect to moisture and fat adsorption, emulsifying capacity, emulsion stability, among others, were noted. Addition of 3% (w/w) shrimp protein hydrolysate to mechanically deboned chicken meat (MDCM) resulted in cooking yield and drip volume of 118% and 5.3 mL/100 g, respectively, as compared with 76% and 10.6 mL/100 g for the control. These effects of the addition of shrimp protein hydrolysates compare favourably with those obtained for commonly used phosphates (sodium tripolyphosphate, trisodium pyrophosphate and sodium hexametaphosphate) in food. The protein hydrolysates also displayed antioxidant activity in a meat model system by inhibiting the formation of 2-thiobarbituric acid reactive substances (TBARS) and in a β-carotene/linoleate emulsion system by delaying the bleaching of β-carotene. Sephadex G-15 column separation of the peptides of shrimp protein hydrolysate

afforded 5 fractions based on their absorbance at 220 and 280 nm, with fraction IV being most antioxidatively active. All fractions were further separated using a reversed-phase high performance liquid chromatographic system and afforded multiple peaks with different retention times based on the hydrophobic nature of the peptides. The eluates also had antioxidative activity when tested in a β-carotene/linoleate system.

The high level of free amino acids and peptides in the shrimp protein hydrolysates was exploited in the generation of volatile flavour compounds from the reaction involving shrimp protein hydrolysate and glucose via Maillard reaction. The compounds obtained included pyridines, pyrazines, aldehydes, furan derivatives, sulphur-containing compounds, among others. The number and concentration of volatile compounds from hydrolysate 1 (DH, 11.2%) and hydrolysate 2 (DH, 17.1%), only differed marginally. The major compounds formed were Strecker aldehydes and their corresponding substituted pyrazines. The most abundant volatile compound formed was 3-methylbutanal which accounted for 28% of the total amount of flavour compounds identified in hydrolysate 2.

Extraction of antioxidant components of shrimp was performed by employing various solvents which differed in their polarity. Ethanol was the most suitable extraction medium and afforded extracts which exhibited antioxidant activity in a β-carotene-linoleate emulsion system. The components of the extract were separated using a silica gel column and further isolation was performed by thin layer

chromatography which afforded four components. Two low polarity compounds as well as two high polarity compounds were separated using n-hexane-acetone (3:1, v/v) and n-butanol-water-acetic acid (3:1:1, v/v/v), respectively, as developing solvents. Reversed-phase HPLC was used to purify the compounds followed by their structural elucidation using electrospray mass spectrometry. The compounds are 3-membered ring phenolics and were identified as 7-(3-butenyl)-2-hydroxy-6-(1,2,5,6-tetrahydro-pyridin-2-yl) quinoline and 7-(3-butenyl)-2-hydroxy-6-(1,2,5,6-tertahydropyridin-2-yl)-7,8-dihydroquinoline and their isomers.

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LIST OF ABBREVIATIONS

AOAC - Association of Official Analytical Chemists

AMU - Atomic mass unit

BHA - Butylated hydroxyanisole

BHT - Butylated hydroxytoluene

BV - Biological value

CE - Capillary electrophoresis

CZE - Capillary zone electrophoresis

DFO - Department of Fisheries and Oceans

DH - Degree of hydrolysis

DNA - Deoxyribonucleic acid

EC - Emulsifying capacity

EDTA - Ethelenediamine tetraacetic acid

ES - Emulsion stability

ESMS - Electrospray mass spectrometry

EPR - Electron paramagnetic resonance

FAO - Food and Agricultural Organization

FPC - Fish protein concentrate

GC - Gas chromatography

GC-MS - Gas chromatography-mass spectrometry

HPLC - High performance liquid chromatography

HPP - Hydrolysed plant protein

HVP - Hydrolysed vegetable protein

LEC - Ligand exchange chromatography

MDCM - Mechanically deboned chicken meat

MS - Mass spectrometry

MSCM - Mechanically separated capelin meat

MW - Molecular weight

m/z - Mass/charge ratio

NR - Nitrogen recovery

OPA - Orthophthalaldehyde

O.D. - Optical density

PAGE - Polyacrylamide gel electrophoresis

PER - Protein efficiency ratio

PG - Propyl gallate

RH - Relative humidity

ROS - Reactive oxygen species

RP-HPLC - Reverse phase-high performance liquid chromatography

RSM - Response surface methodology

SAS - Statistical Analysis System

SD - Standard deviation

SDS - Sodium dodecyl sulphate

SEC - Size exclusion chromatography

SEC-HPLC - Size exclusion high performance liquid chromatography

SHMP - Sodium hexametaphosphate

SHPH - Shrimp protein hydrolysate

STPP - Sodium tripolyphosphate

TBA - 2-Thiobarbituric acid

TBARS - 2-Thiobarbituric acid reactive substances

TBHQ - Tertiary butylhydroquinone

TCA - Trichloroacetic acid

TFA - Trifluoroacetic acid

TLC - Thin-layer chromatography

TNBS - 2,4,6-trinitrobenzene sulphonic acid

TSPP - Tetrasodium pyrophosphate

UNU - United Nations University

USDA - United States Department of Agriculture

UV - Ultra violet

WHC - Water holding capacity

WHO - World Health Organization

CHAPTER 1

INTRODUCTION

Seafoods form a major component of human diet in various parts of the world. Although the world seafood harvest is estimated at over 100 million metric tons, only about a fifth of this is used for human food (FAO, 1996). It is, however, estimated that over 60%, of industrially processed fish for human consumption are by-products (Raa and Gildberg, 1982). Efforts have been made in the past decades to better utilize the resources from the oceans. In addition to the wastes, there are unutilized species which are not desired for direct consumption due to certain characteristics which such species possess.

Some techniques have therefore been developed in the past decades for utilization of these species, including fish meal, silage, fish sauce, protein concentrate and hydrolysate preparation. Furthermore, attempts have been made to find alternative methods of harnessing the easily digestible and nutritionally rich proteins of aquatic species. Production of thermostable protein dispersions is one of such methods that is being exploited in our laboratory (Venugopal and Shahidi, 1994). The process, which basically involves the aqueous extraction of pigments, sarcoplasmic proteins and odorous matter, results in the production of concentrated myofibrillar proteins which when homogenized with or without addition of an organic acid gives a highly viscous preparation that is stable to heat treatment and have comparable amino acid composition as the original meat. Such protein dispersions may be used in a variety of food formulations.

In recent years, significant strides have been made toward recovery of protein and other valuable components from seafood processing discards and underutilized species. Enzymatic protein hydrolysis is one method of choice that has been applied to both animal and plant proteins (Adler-Nissen, 1986a; Shahidi *et al.*, 1995). However, not all seafood processing discards and underutilized aquatic species are being utilized to their full potential. The protein hydrolysis method has the advantage of being able to control the experimental conditions and hence obtain hydrolysates with desired physicochemical characteristics. Response surface methodology (RSM) is an important technique that has been used for studying complex processes, especially in the food industry, in order to optimize food science-related process operations (Thompson, 1982; Joglekar and May, 1987; King and Zall, 1992; Baek and Cadwallader, 1995; Wanasundara and Shahidi, 1996). RSM consists of a group of mathematical and statistical procedures which can be used to assess the relationship between a number of factors (independent variables) and one or more responses (dependent variables). The methodology also generates a mathematical model that adequately describes the overall process. This procedure may be applied to optimize the preparation conditions of protein. Proteins so obtained, apart from having unique functional characteristics that make them suitable ingredients in a variety of food formulations, may also contain high levels of free amino acids which are flavour-active components, and hence may offer potential use as components of bait. Thermal treatment of peptides with reducing sugars can generate more specific flavour

compounds, which give characteristic aromas (Rizzi, 1989; Oh *et al.*, 1992). Thus protein hydrolysates may be used as ingredients for thermal flavour generation, typically through the Maillard reaction (Ho *et al.*, 1992).

The antioxidant activity of amino acids, peptides and proteins has been reported (Marcuse, 1962; Ahmad *et al.*, 1983; Decker *et al.*, 1992; Lin *et al.*, 1993; Chen *et al.*, 1995; Amarowicz and Shahidi, 1997). The presence of certain amino acids is believed to be essential for a particular peptide to possess antioxidant activity and hence the fractionation of peptides on the basis of their hydrophobicity may give a clue about the nature of amino acids involved. High performance liquid chromatography, in the reverse-phase mode, has been widely used for isolation and subsequent characterization of peptides present in protein hydrolysates (Perea *et.*, 1993; Reid *et al.*, 1994; Chen *et al.*, 1995).

The presence of antioxidant components in shrimp was first noted by the extension of shelf life of seafood preparations containing shrimp, as compared with controls devoid of such components (Babbitt *et al.*, 1974). Further studies have since been carried out which point to the presence of antioxidant compounds in shrimp. The nature and chemical structures of these compounds have been mainly speculative. Appropriate solvents may be used for the extraction of the antioxidant compound(s), which may be followed by purification by suitable chromatographic techniques. The purified compounds may be subjected to appropriate spectroscopic techniques to elucidate their chemical structures. One of such techniques is the electrospray mass

spectrometry (ESMS), which is one of the softest ionization methods, which requires neither derivatization nor excessive manipulation of the analytes (Banoub *et al.*, 1997). Electrospray ionization is well established as a robust technique, which allows the rapid, and sensitive analysis of a wide range of analytes from low molecular mass polar compounds (<200 Da) to biopolymers >500 kDa (Fenn *et al.*, 1989). The technique has been extensively used for structural elucidation of organic compounds (Gentil *et al.*, 1994; Masuda and Jitoe, 1994; Gentil and Banoub, 1996).

The hypotheses of this study and the experimental approach (objectives) to test them were:

1) Many fish and other aquatic species are underutilized and contain recoverable proteins in their tissues or processing discards. Hence the first set of objectives were to a) prepare protein dispersions which are thermostable and highly viscous from capelins and shark meats, b) prepare protein hydrolysates from shrimp heads and female carcasses of lumpfish after roe extraction using optimum hydrolysis conditions obtained by response surface methodology, and c) determine the functional and antioxidative properties, as well as nutritional value of protein hydrolysates prepared.

2) Protein hydrolysates have high levels of free amino acids which are taste active and are also precursors of certain heat-processed flavours. Hence the second objective was to generate thermal flavour compounds using shrimp hydrolysates and glucose and to characterize the types of compounds thus formed and quantify their contents.

3) Shrimp has phenolase and other phenolic degrading enzymes and hence it might be safe to hypothesize the presence of phenolic compounds in shrimp which may have antioxidative properties. Therefore, the third objective was to isolate and characterize the antioxidant compounds present in shrimp.

CHAPTER 2

LITERATURE REVIEW

2.1 Seafoods - General Introduction

Seafoods constitute a tangible part of protein source available to humans in many countries. The world's annual catch of fish and marine invertebrates is approximately 110 million metric tons, from which only 20% is processed for food use (FAO, 1996). Approximately 30% of the latter amount is consumed and the rest is generally discarded. The harvest of seafoods from aquaculture is over ten million tons which is in addition to the convectional catch (Sikorski and Pan, 1994). The utilization of different species of fish and invertebrates is affected by their size, high bone and/or fat content, flavour and colour. Thus, many species of fish and crustaceans such as sturgeon, salmon, grouper, crab and lobster are highly valued because of their perceived superior image. On the other hand, other species such as, capelin, menhaden and lumpfish remain underutilized and are generally reduced to fish meal and oil or discarded (Sikorski and Pan, 1994). However, extraction of roe from several species for production of caviar-type commodities is practised. Different methods have been suggested in order to utilize low-value fish. These methods include use of exogenous enzymes or utilization of enzymes already present in the source material in order to hydrolyse the proteins (Haard, 1994). Production of functional proteins via repeated washing of the minced fish is also possible (Lee, 1994). Seafood proteins are important in that they possess a well-balanced amino acid composition and are also easily digestible (Kent, 1987).

2.1.1 Seafood proteins

Proteins generally constitute approximately 11-27% of seafoods and are usually classified as sarcoplasmic, myofibrillar and stroma. The sarcoplasmic proteins usually refer to those of the sarcoplasm, the components of the extracellular fluids, and these proteins are contained in small particles of sarcoplasm. The sarcoplasmic proteins, which are the water-soluble protein components include albumin, myoglobin and enzymes, account for approximately 30% of the total muscle protein. The content of this protein type is generally higher in pelagic fish species, such as sardine and mackerel, than in demersal fish, such as cod and halibut. White muscle of certain species contains more sarcoplasmic protein as compared with those of their dark muscle counterparts (Suzuki, 1981). The truly intracellular soluble fraction makes up 90 to 95% of the total proteins extracted by homogenizing the muscle tissue with water or solutions of neutral salts at an ionic strength usually below 0.15 Debye (Scopes, 1970). Among the sarcoplasmic enzymes influencing the quality of fish as a food item are mainly the enzymes of the lysosomes (Sikorski *et al.*, 1990).

The myofibrillar proteins in muscle are myosin, actin, actinomyosin and troponin and these account for 40-60% of the total crude protein content of fish. These proteins undergo changes during the rigor mortis, resolution of rigor and long-term frozen storage. These changes affect solubility of fish muscle proteins (Stefansson and Hultin, 1994). Aqueous washing of muscle results in the removal of mostly sarcoplasmic proteins, thereby concentrating the myofibrillar components in

the washed samples (Onodenalore, 1993). The washing process also removes odoriferous substances from aquatic species, especially when a bicarbonate solution is used (Shahidi and Venugopal, 1994). The texture of fish mince and surimi is affected by aqueous washing of fish muscle proteins. The myofibrillar fractions of muscle proteins dictate the general functional characteristics of tissues. The myofibrillar proteins are intermediate in solubility and influence the culinary properties of the fish meat because of their high water-binding and emulsifying capacities (Asghar *et al.*, 1985). The solubility of muscle proteins has been of interest due to the relationship of solubility with functional properties such as gelation, emulsification, water uptake, binding strength and thermal properties (Hultin *et al.*, 1995). In surimi processing, extensive washing is utilized to remove water-soluble substances, mainly sarcoplasmic proteins. In the process, the myofibrillar proteins are concentrated and these are responsible for the gel-forming ability of surimi (Okada, 1964); this is a desired characteristic of surimi in the formulation of products such as kamaboko.

Myofibrillar proteins are generally classified as salt-soluble proteins because high salt concentrations are required to solubilize them. However, studies have shown that a considerable amount of myofibrillar protein is lost in surimi waste streams (Lin *et al.*, 1995). The loss of these proteins during surimi processing could be, in part, due to their water solubility. Hultin *et al.* (1995) reported that some of the myofibrillar proteins are soluble in solutions of relatively low ionic strengths. Studies

have further revealed that the proteins of cod muscle and chicken breast are almost completely soluble in solutions of physiological ionic strength or less (Stefansson and Hultin, 1994). The muscle proteins dissolved spontaneously when the minced muscle was washed with a solution of sufficiently low ionic strength. In case of chicken breast muscle, however, repeated washing of the muscle tissue to an ionic strength lower than that required for solubilization of cod muscle proteins did not have a similar effect on solubilization in water of most of its proteins (Krishnamurthy *et al.*, 1996). This indicates inherent characteristics in different muscle types which may affect their solubility profile. Hennigar *et al.* (1989) also reported that gels could be prepared using fresh muscle without NaCl, suggesting that fish muscle myofibrillar proteins could be quite soluble in water or low ionic strength solutions. Lin and Park (1996) recently reported that sarcoplasmic proteins were readily extracted with water during washing of minced pacific whiting and thus myofibrillar proteins became relatively soluble and hence lost during extensive washing. Interest has focused on myosin and actomyosin components of myofibrillar proteins since the functional properties of muscle proteins are believed to be dominated by them.

Connective tissue proteins, collagen, elastin and reticulum, constitute a third important group of muscle proteins also referred to as stroma proteins. These proteins are soluble in dilute solutions of HCl and NaOH and contribute up to 10% of the crude muscle proteins. Their major function as constituents of muscle, bone, tendons and ligaments is one of support. Although they are extracted by salt solutions, these

proteins have poor emulsifying ability and gelatinize during cooking (Aberle and Mills, 1983).

Non-protein nitrogenous (NPN) compounds are also present and their levels depend on the species as well as the muscle type under consideration (Shahidi, 1994a). The NPN content in seafood is generally higher than that in other food sources, which is in the range of 9-18% of total nitrogen for teleosts and 33-38% for elasmobranchs (Belitz and Grosch, 1987). The dark muscle of fish generally contain a higher amount of NPN as compared with light muscle. The major classes of these compounds include free amino acids, peptides, guanidines, urea and nucleotides, among others. The NPN compounds are important for their taste effect in seafoods (Haard *et al.*, 1994).

2.1.2 Functional seafood proteins

Proteins, either in modified or unmodified forms, may be used as functional ingredients in food products (Kinsella, 1982). Furthermore, fish proteins may be used to enhance the nutritive value of cereal-based foods and for production of animal feed. However, such efforts are hampered due to the intrinsic nature of fish muscle proteins such as lack of solubility in water as well as sensitivity to denaturation (Venugopal and Shahidi, 1994). Fish myosin and actomyosin are the components generally regarded as being sparingly soluble in water or low-salt solutions at neutral pH values. This may be due to the tendency of myosin molecules to interact with one

another under physiological conditions while in the presence of several low-molecular-weight compounds and enzymes that remain adhered to them in their native state (Suzuki, 1981; Nakagawa *et al.*, 1989).

Many functional properties of muscle food proteins are related to their solubilization in salt or aqueous solutions. Fish proteins are known to be very unstable as they undergo rapid denaturation with associated loss of solubility and functional properties, depending on the processing conditions. Solubility of proteins is correlated with functional properties such as emulsifying properties, foaming capacity and gelation (Kinsella, 1976, 1982). The widely accepted concept of the formation of fish gels has been that a high concentration of sodium chloride is required to solubilize myofibrillar proteins (Suzuki, 1981; Lee, 1984, 1986; Shimazu, 1985). The proteins present in both non-utilized or underutilized fish species may be used in various ways, in addition to preparation of surimi-type products.

2.2 Underutilized and non-utilized aquatic species

2.2.1 Lumpfish

Lumpfish (*Cyclopterus lumpus*) is a semi-pelagic fish belonging to the family *Cyclopteridae*, a group of small stout-bodied fish whose pelvic fins are modified to form an adhesive disc which enables them to adhere to the ocean bottom or other objects. They are found abundantly in the coastal waters of Newfoundland and as far north as the Nain region of Labrador where they were once considered a nuisance, but

now a valuable source of income (Lynch, 1984). From a modest harvest of 21 tons of roe in 1970, the lumpfish roe fishery in Newfoundland has expanded to 1,500 tons in 1996 (DFO, 1997). Lumpfish has been used in the past in a scanty manner for various purposes, ranging from food for dogs and pigs, as bait for lobster traps, and for human consumption (Collins, 1976). Female lumpfish are harvested solely for their roe which has its primary markets in Europe and Japan where the roe is processed as caviar, which is considered a delicacy. There is virtually no demand for lumpfish carcasses in the fish meal industry because of their high water content and low protein and oil yield (Davenport, 1985). It is a common practise for fishermen to extract the roe sacs from female lumpfish and discard their carcasses as well as any males harvested.

The amount of roe harvested in Newfoundland and Labrador in 1996 was 1.5 million kilograms which translates to approximately 6 million kilograms female carcass weight, based on a 25% roe yield, as well as perhaps an equal weight of male carcasses. However, the potential use of lumpfish skin or carcass for production of gelatin has been considered (Osborne *et al.*, 1990). Gelatin is a protein material obtained by the hydrolysis of collagen which is the main component in the skin and connective tissues of mammals and fish and acts as the binder in the bones of vertebrates (Glicksman, 1969). An acid extraction process was employed for extraction of gelatin from lumpfish skin as well as from headed and gutted lumpfish. The headed and gutted lumpfish served best as a source of gelatin (Osborne *et al.*,

1990). Although traditional lumpfish caviar has been prepared from cured roe, with a salt content of 13 to 15%, studies have been carried out using alternative methods involving washing of the fish roe in brine, addition of both dry and liquid ingredients to the roe, and a final rinse step to ensure that excess residual ingredients were not left on the product (Power and Voigt, 1990). This procedure yielded a high quality product when fresh roe was processed rapidly after harvesting, pasteurized and stored under appropriate conditions. However, little attention has been paid to the utilization of whole male lumpfish and female carcasses after roe extraction. In Iceland, however, edible lumpfish preparations have been developed. There is also a small domestic market for lumpfish meat in Europe where it is a favoured food in some areas (Anon., 1971).

The total lipid content of the male flesh and female (roe removed) lumpfish, on a dry weight basis, averaged 3.9 and 1.9%, respectively. The difference in the lipid content was presumed to be due to the transfer of lipid from muscle and liver to roe in females during spawning and the period preceding it (Paradis *et al.*, 1975).

2.2.2 Shellfish processing discards

The term 'shellfish' includes both molluscs and crustaceans and a few other invertebrates such as sea urchins. They are characterized by the presence of a hard exoskeleton, which is not usually consumed directly, and may be discarded following processing. However, there are exceptions, such squid and octopus which lack the

hard exoskeleton. Over the past two decades the shellfish industry has experienced a significant increase in processing volume with a corresponding increase in the available by-products, most of which are not presently utilized. The waste from shellfish processing plants is a serious concern for processors if strict environmental regulations are enforced. Different species of shellfish are mainly processed in large facilities world-wide, each variety with production rate in order of several thousand tons per year (Hansen and Illanes, 1994).

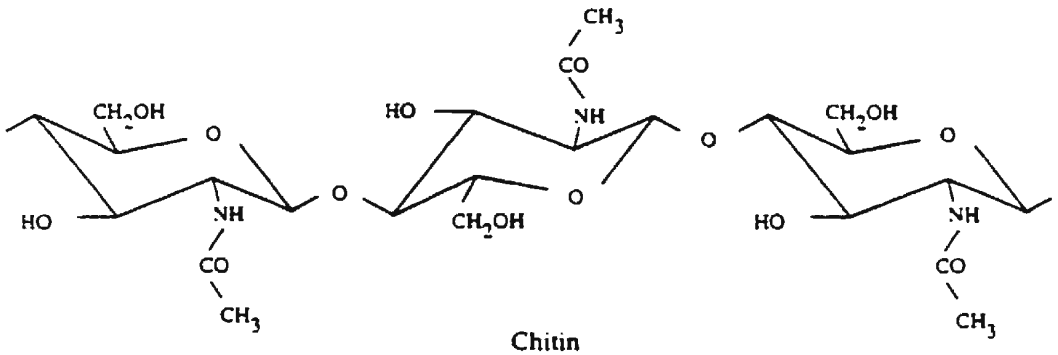
The most important commercially harvested crustaceans in Canada are crab, shrimp and lobster. In the crustacean industry, processing discards may account for up to 80% of the landed catch. These materials are mainly shells, viscera, heads and adhered meat. The major components of shells are proteins, chitin and minerals, as well as small amounts of flavourant proteins and carotenoid pigments (Hansen and Illanes, 1994; Shahidi *et al.*, 1992a; Ferrer *et al.*, 1996). The techniques which are available for disposal of processing by-products include ocean dumping, incineration or disposal to landfill sites (Revah-Moiseer and Carroad, 1981; Shahidi, 1994a). However, environmental restrictions and a better understanding of potential value of processing discards for a variety of applications have resulted in efforts to find uses for these materials. There has also been a growing interest in the natural ingredients which are available from shellfish discards. The processing of shellfish wastes poses a major technological problems, as shells are largely water-insoluble and very resistant to natural biodegradation (Healy *et al.*, 1994). The constituents of such shells include

approximately 13-42% protein and 14-30% chitin which are worthy of further processing. Crab and shrimp fishery are of utmost importance to the economy of Newfoundland. Therefore, value-added utilization of their discards is of importance. Crab wastes contain 17-32% chitin, whereas that of shrimp wastes is 14-27%, on a dry weight basis (Shahidi and Synowiecki, 1991; Ferrer *et al.*, 1996).

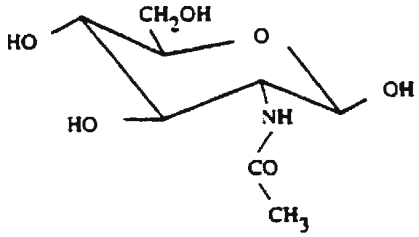
2.2.2.1 Chitin, Chitosan and pigments from shellfish processing by-products

Chitin is a carbohydrate-based polymer which is second in abundance only to cellulose in nature (Austin *et al.*, 1981). It is found in the exoskeleton of crustaceans, insects and mushrooms. The monomer of chitin, N-acetyl-D-glucosamine units, are joined together via β(1-4) linkages. Chitosan is the deacetylated (to different extent) form of chitin (Shahidi and Synowiecki, 1992). The chemical structures of chitin, its monomer N-acetyl-D-glucosamine, and chitosan are shown in Figure 2.1. Over the past two decades, attention has been focused on the separation of different components of shells using physical techniques (Muralidhara and Maggin, 1985), application of enzymes to hydrolyse their protein and thus recovering chitin (Gagne and Simpson, 1993), as well as use of bioconversion processes to modify the component fractions, particularly the chitin to its monomer. Commercially, crustacean shells are used for the extraction and isolation of chitin and its subsequent conversion to chitosan by alkali treatment. There are high concentrations of chemicals (acid or alkali) and high energy input for the deproteination stage. Chitin is insoluble in almost every common

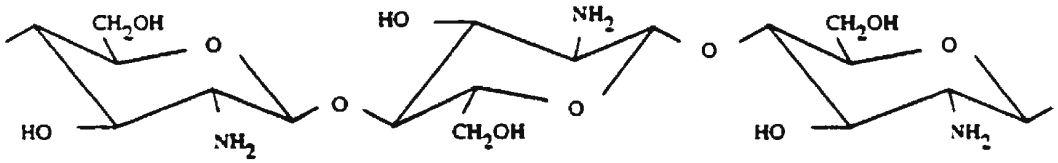
Figure 2.1 Structures of chitin, acetyl-D-glucosamine and chitosan .



Chitin



N - Acetyl - D - glucosamine



Chitosan

organic solvent and in acids, basic and neutral aqueous solutions. However, chitosan is soluble in acid solutions.

In addition to associated adverse environmental and economic aspects, use of acid or alkali does not allow the recovery of other value-added products present in the shells, such as proteins and pigments. The carotenoids, mainly asthaxanthin and its mono- and diesters, may serve as a natural source of pigment for inclusion in the feed for salmonids. In the wild, salmonids ingest crustaceans and other sources of carotenoids, hence acquire their desired colour. However, pigments must be added to the diet of farmed salmon, trout and char in order to achieve pigmentation.

On a world-wide basis, several million tons of crustacean shells are generally disposed on an annual basis. Two main approaches for the utilization of chitin/chitosan exist. One, the bioconversion of chitinous polymers to microbial biomass proteins by use of the yeast, *Picia kudriarvenvii* which grows on hydrolysates obtained from chitin by chitinase digestion (Carroad and Tom, 1978; Cosio *et al.*, 1981). The other method is utilization of chitin/chitosan for the removal of other water contaminated waste materials such as heavy metals (Muzzarelli *et al.*,1989), dyes (Vankatrao *et al.*, 1986), pesticides (Thomé and Van Daele, 1986), amino acids (No and Meyers, 1989) and proteins (Knorr, 1991). Chitosan could also be used for the removal of microorganisms (Popper and Knorr, 1990) and production of biodegradable packaging films (Mayer *et al.*, 1989). It is also used as an ingredient in cosmetics and pharmaceuticals. Chitin/chitosan application in enzyme and cell

immobilization, drug delivery, seed coating, among others, has been documented (Sandford, 1989; Muzzarelli *et al.*, 1989). A list of current uses of chitin and its derivatives is given in Table 2.1.

2.2.3 Shrimp waste

The shrimp industry, through various processing operations, produces a large amount of potentially recoverable proteinaceous by-product in the form of heads and shells as well as a variety of valuable dissolved and particulate organic matter (Meyers, 1986). Shrimp heads constitute approximately 30-40% of the weight of the animal and these may be readily recovered as proteinaceous products for use in feed formulations. Shrimp by-products are of interest as potential flavour components of processed seafood products as well as livestock feed formulations, especially for farmed shrimp. The shrimp heads may be readily processed to meal and silage (Fox *et al.*, 1994; Fagbenro and Bello-Olusoji, 1997).

Shrimp meal production requires fresh raw material in view of the active proteolytic enzymes and microbial degradative processes that may occur, and would result in poor quality meals with significant loss of protein and short storage life (Meyers, 1986). Production of low-fibre and low-chitin pulp from shrimp heads by cooking the heads to inactivate enzymes and denature proteins, followed by pressing to reduce water content, has been reported (Barrat and Montaño, 1986). The product obtained is finally passed through meat/bone separator and the wet meal may be

Table 2.1 Current practical uses of chitin, chitosan and their derivatives[1].

Uses	Compounds
Cationic sludge dewatering and flocculating agents for polluted waste waters	Chitosan
Recovery of metal ions and proteins in aqueous waste solutions	Chitosan
Agricultural material (e.g., plant seed coating, fertilizer)	Chitin and chitosan
Food additives (e.g., as stabilizers, thickeners, inhibitors of lipid oxidation)	Chitin and chitosan
Feed additives for pets, fishes and animals	Chitin and chitosan
Food processing (e.g. sugar refining)	Chitosan
Hypocholesterolemic agents	Chitosan
Dressing materials for burns and skin lessions of humans and animals and for plant tissue wounds	Chitin and chitosan
Biomedical materials (e.g. adsorbable suture)	Chitin and N-acylchitosans
Blood anticoagulant materials	Sulphated chitin
Blood antithrombogenic materials	N-Hexanoyl and N-octanoyl derivatives
Homostatic materials	Chitosan
Cosmetic indredients for hair and skin cares	Chitosan and chitin
Textile and woven fabrics	Chitin, chitosan, chitin xanthate
Paints and dyeing and weaving	Chitosan
Natural thickeners	Chitosan
Papers, films, sponge materials	Chitin and chitosan
Chromatographic and immobilizing media	Chitin and chitosan
Biotechnology (e.g., enzyme immobilization, encapsulation)	Chitin

[1] Shahidi (1995) and Hirano (1997).

frozen or dried for further processing or storage. Such pulp has a moderate protein content (12.5%), is relatively low in ash and chitin (6.5%), and has been readily accepted by shrimp brookstock.

Shrimp silage is another product which can be obtained from shrimp heads through liquefaction of shrimp heads via the addition of organic acids which possess antiseptic and bacteriostatic properties. This is a cheap technique which will stabilize and retain the nutritional quality of this resource for aquaculture feed. The resulting liquid silage could be blended with fillers (dry protein feedstuffs) to aid drying, but the choice is determined by cost and local availability of such raw material (Lopez, 1990). Dried shrimp head silage has been blended with alternative fillers such as hydrolysed feather meal, poultry by-product meal or soybean meal. Such preparations have been incorporated as protein supplements into pelleted semi-purified diets for catfish (Fagbenro and Bello-Olusoji, 1997). High apparent digestibility coefficient of dry matter and crude protein in the silage by catfish fingerlings was obtained which indicates that the product is suitable as feed and has a potential as a protein feedstuff in fish diets.

Apart from residual protein and minerals the other major component of shrimp shell waste is chitin. In its natural form, a broad range of partially deacetylated polymers exists. In practice the polymer, where most residues are acetylated, is called chitin while the opposite is true for chitosan (Hansen and Illanes, 1994). Several processes have been proposed for the fractionation of shellfish waste and production

of chitin and chitosan. Most processes for chitin production include alkaline extraction of proteins and dilute acid solubilization of minerals. Production of chitosan follows simple unit operations of deacetylation, washing and drying (Shahidi, 1995).

2.3 Recovery of protein in seafood processing discards and underutilized species

The processing by-products and underutilized/non-utilized species are good sources of protein and account for several million tons of raw material which may be harvested for humans and other valuable uses by employing appropriate techniques. A major component of seafood processing discards and underutilized species is crude protein. Attempts have been made to recover these proteins by various processes which may be enzymatic or non-enzymatic in nature. This may lead to the production of fish sauce, silage, fish meal, protein concentrate and protein hydrolysate. The appropriate method for processing of such material depends on the nature of raw material, the desired characteristics of the product and its intended use.

2.3.1 Fish sauce

Fermented fish products have long been produced in South-East Asia; fish sauce which is a major fermented fish product was known in ancient Greece and Rome (Amano, 1962; Corcoran, 1963). Fish sauce is made by heavily salting small fish or fish waste for a period of several months, during which the endogenous

digestive enzymes hydrolyse most of the proteins, thus yielding an aqueous solution rich in short-chain peptides and free amino acids (Orejana and Liston, 1982). The annual production of fish sauce in South-East Asia is over 250,000 tons where it is used as a condiment on rice dishes (Stephanson and Steingrimsdottir, 1990). The addition of enzymes in fish processing probably first occurred in Vietnam, where one of the traditional fish sauce products is made by mixing enzyme-rich pineapple juice with eviscerated fish and salt (Owens and Mendoza, 1985). Industrial production of fish sauce is fairly large in Asia. However, its production has been extended to other parts of the world, including North America.

2.3.2 Fish Silage

Fish silage is a more recent invention which has many characteristics in common with fish sauce. It is produced by grinding fish processing discards or by-catch followed by acidification which preserves the material and provides the appropriate pH condition for endogenous digestive enzymes to degrade proteins. Various acids and acid mixtures have been used, but formic acid is most commonly used. The aqueous product obtained may be partially dehydrated to give silage which is used as a source of protein in feed products and possibly for soil enrichment. For a rapid hydrolysis of silage with low protease activity, it may be necessary to add enzymes or enzyme-rich raw material. However, if silage is made from fish viscera, which are very rich in proteolytic enzymes, the autolysis will proceed rapidly without

the addition of exogenous enzymes (Freeman and Hoogland, 1956). Fish silage was first made in Scandinavia during the Second World War (Petersen, 1953). The silage preparation method, where the separation of oil and protein-rich fractions is promoted by the action of endogenous enzymes may be considered as an alternative to fish meal production.

2.3.3 Fish meal

Fish meal may be produced from different species of non-utilized and underutilized fish, especially those with high oil content or from fish offal, scraps and cannery wastes. The production requires cooking followed by pressing to remove most of the oil and some water. The moisture level in the meal is reduced by drying, which retards microbial spoilage and enhances stability of the product. The press cake is then ground and may be further stabilized by addition of antioxidants such as ethoxyquin (Shahidi, 1994c), particularly for meals made from high lipid-containing fish. Fish meal has been used for many years as livestock feed (Windsor, 1982). The meal is popular because of its high nutritive value, if properly processed. It contains high levels of essential amino acids such as lysine, which is often deficient in grain products which form the basis of most animal feeds. In addition, it contains an adequate supply of vitamins such as cyanocobalamine, choline, niacin, pantothenic acid and riboflavin, as well as minerals, namely calcium, copper, iron, phosphorous and other trace elements (Ockerman, 1992). Fish meal is also low in fibre and easy

to produce. There are two basic types of fish-meal production techniques; wet and dry rendering. The wet rendering method is usually used when there is large supply of raw material to be rendered and in this procedure, the fish are disintegrated, boiled, deboned, separated, pressed and partially dewatered (12 to 20% moisture). The raw material at this point may be preserved in 15 to 25% short chain alcohols which would also be normally used in subsequent extraction. The procedure is very useful with fatty fish and produces not only fish meal, fish solubles and fish scraps, but also fish oils (Ockerman, 1992).

The dry-rendering method is usually used for non-fatty fish such as haddock, cod cannery material, shark or grayfish carcasses. The procedure involves grinding and then cooking in a steam jacket cooker. The meal is then pressed to remove the liquor and the water-insoluble fraction remains with the press cake. Long cooking periods (6 to 7 h under pressure) and the high temperatures used for rendering, usually result in a dark coloured low quality oil. Other manufacturing procedures are solvent extraction such as acid, base, hydrogen peroxide or sulphur dioxide, which are sometimes used with subsequent removal of the solvent.

2.3.4 Protein concentrate

The production of fish protein concentrate (FPC) is achieved by solvent extraction of fat from comminuted whole fish or by-products. The most commonly used extractants are ethanol and isopropanol. Following solvent recovery, the meal

is dried, milled and screened for removing large bone particles (Doe and Olley, 1990). Taints develop easily in the product due to bacterial action and active proteolytic enzymes in raw material as well as the presence of highly unsaturated fatty acids which oxidize rapidly. Due to its poor functional properties and residual fishy flavour, FPC is not accepted as a food additive. Unmodified protein concentrates have an extremely low water affinity and impart a gritty and powdery texture to products. However, their functional properties may be improved using chemical modification or partially controlled proteolysis (Sikorski and Naczk, 1981). Based on the quality of the final product, such concentrates are grouped into 3 classes (Mackie, 1983): Type A, which is a deodorized and tasteless product containing less than 0.75% fat and a minimum of 67.5% protein; Type B, this is a hygienically prepared fish meal with a maximum of 3% fat; and type C which is the usual fish meal.

2.3.5 Protein hydrolysates

Protein hydrolysates are produced by hydrolytic breakdown of proteins through enzymatic or chemical means to produce highly soluble low-molecular-weight compounds which are mainly free amino acids and peptides. Protein hydrolysates are produced for two distinctly different purposes: i) enhancement of desirable functional properties and ii) production of small peptides and amino acids which are amenable to use in dietetic foods and as flavouring agents or precursors (Kilara, 1985). Protein hydrolysates have been used as food ingredients for over 80 years and their unique

properties have been rediscovered within the past 30 years (Mahmoud, 1994).

The term protein hydrolysis applies to all possible ways of breaking the peptide bonds which link individual amino acids together in a peptide or protein to produce lower molecular weight compounds such as short chain peptides and free amino acids. The 20 common amino acids which are the building blocks of proteins vary in their molecular weights and chemical properties. Some of these bonded amino acids still have reactive groups which are either polar, reductive or hydrophobic in nature. These groups interact with those of neighbouring peptide chains to form three-dimensional structures (Lieske and Konrad, 1994). During hydrolysis. the proteins with large molecular size are attacked at many peptide linkages along the chain to produce a variety of breakdown products, as given below.

Protein ⇒ primary proteoses ⇒ peptones ⇒ polypeptides ⇒

simple peptides ⇒ free amino acids

Some of these degradation products contribute in various ways to the useful functional properties of protein hydrolysates, such as physicochemical, dietetic as well as nutritive properties and flavour notes. There are three ways to hydrolyse proteins; these are i) fermentation, ii) chemical hydrolysis, which involves use of strong acid or base, and iii) enzymatic hydrolysis by use of appropriate proteases. Fermentation is generally not feasible because of the slowness of the process, thus allowing the growth of spoilage or pathogenic microorganisms, hence it is the least preferred method to hydrolyse proteins. The chemical and enzymatic methods are more commonly used.

2.4 Methods of hydrolysis

2.4.1 Chemical hydrolysis

Chemical hydrolysis involving the use of acid is more commonly practised as compared with that of base. Acid hydrolysis is relatively low in cost and is an efficient and rapid method. Protein hydrolysate can be made from a variety of proteinaceous material. Generally, all proteinaceous material can be hydrolysed. Plant proteins are commonly used and the resulting products are designated as hydrolysed plant proteins (HPP) or hydrolysed vegetable proteins [HVP] (Manley *et al.*, 1981). Many acids, including carbonic, formic, acetic and phosphoric acids are possible hydrolytic agents (Lieske and Konrad, 1994). However, hydrochloric acid is commonly used and this is usually added to the material to be hydrolysed and heated at 100 - 125 °C followed by neutralization with sodium hydroxide or another suitable base. The solubilized mixture is decolorized and partially deodorized by filtration through activated charcoal (Manley *et al.*, 1981), then concentrated and finally dehydrated, usually by freeze-drying or by spray-drying. Acid hydrolysis has several disadvantages which may preclude its use as an ideal method for production of protein hydrolysate. Some amino acids such as methionine, threonine and tryptophan are partially destroyed and high levels of salt is formed during neutralization of the hydrolysed products (Grace, 1974). Racemization of some amino acids would lead to the formation of their D-forms and also sharp tastes. The acid

hydrolysis, however, has the merit of extensively degrading proteins and breaking peptide bonds which would not be hydrolysed by the enzymatic process.

2.4.2 Enzymatic hydrolysis

2.4.2.1 Enzymes in hydrolysis

Enzymes are proteins with the capacity to catalyse specific biochemical reactions. They are thermolabile, of relatively high molecular weight, and synthesised by living cells in which they catalyse the vast number of reactions that constitute cell metabolism. Enzymes are classified into six major groups according to the reaction type they catalyse (Table 2.2). However, mainly hydrolases have been commonly used in the industry, particularly in food processing, detergent production and biotechnology (Hall and Ahmad, 1992). Hydrolases are responsible for the hydrolytic cleavage of bonds in proteins, lipids and carbohydrates. In production of protein hydrolysates, the hydrolases (usually called proteases or peptidases) are used exclusively in proteolytic processes. A list of commonly used proteases in food is given in Table 2.3. Proteases may be classified according to their source (animal, plant or microbial), their mode of action (endo- or exo-type), and the nature of the catalytic site (alkaline protease or serine protease, sulphydryl protease or cysteine protease, metal-containing protease or metalloprotease, and acid protease) (Adler-Nissen, 1986a; Whitaker, 1994). Endo-type proteases (endopeptidases) hydrolyse peptide bonds randomly within proteins to produce a relatively large peptide chains,

Table 2.2 Enzyme classification system and examples of enzymes used in food and flavour industry.

Class	Types of reaction catalyzed	Examples
Oxidoreductases	Transfer of electrons (hydride ions or H atoms) may require NAD(P)H as cofactor.	Catalase, glucose oxidase, lipoxygenase.
Transferases	Transfer of functional groups such as acyl, glycosyl and phosphate from one substrate to another.	Catecholmethyltransferase
Hydrolases	Hydrolysis reactions (transfer of functional groups to water). Substrates include amides, esters, epoxides, glycosides and peptides.	Amylases, cyanidase, esterases, (hemi)-cellulases, glucanases, glucosidases, (phospho)-lipases, pectinases, proteases, saccharases, ureases.
Lyases	Addition of groups to double bonds or formation of double bonds by removal of groups.	Hydroperoxide lyases.
Isomerases	Transfer of groups within molecules to yield isomeric forms.	Glucose isomerase
Ligases	Formation of C-C, C-S, C-O, or C-N bonds by condensation reactions coupled to ATP cleavage.	

Table 2.4 Typical functional properties performed by functional protein in food systems[a].

Table 2.3 Commonly used proteases in food systems[a].

Common name	Source	Nature of active site	Specificity
Animal enzymes			
Chymotrypsin	Bovine, porcine pancreas	Serine protease	Phe, Tyr, Trp
Pancreatin	Bovine, porcine pancreas	As for trypsin, chymotrypsin	Very broad specificity
Trypsin	Bovine, porcine pancreas	Serine protease	Lys, Arg
Pepsin	Bovine, porcine stomach	Aspartic protease	Aromatic amino acids
Chymosin	Calf abomasum	Aspartic protease	Phe-Met in casein
Plant enzymes			
Bromelain	Pineapple	Cysteine protease	Lys, Arg, Phe, Tyr
Ficin	Fig	Cysteine protease	Phe, Tyr
Papain	Papaya	Cysteine protease	Lys, Arg, Phe
Bacterial enzymes			
Subtilisin	*Baccillus subtilis*	Serine protease	Very broad
Alcalase	*Baccillus licheniformis*	Serine protease	Very broad
Fungal enzymes			
Rennilase	*Mucor miehei*	Aspartic protease	As for rennet
Protease	*Aspergillus spp.*	Aspartic protease	Very broad

[a] Hall and Ahmad (1992).

while the exo-type (exopeptidases) cleaves peptide bonds one by one from terminal amino acids (either carboxyl or amino group).

Serine protease is usually an alkaline protease and active in the pH range from 7.0 to 11.0, and has an essential seryl group in its active site (Kelly and Fogarty, 1976). The sulphydryl protease (or cysteine protease) has essential cysteinyl and histidyl residues in its active site, and exhibits optimum activity at neutral pH. The metal-containing proteases (or metalloprotease) require divalent metal ions, and thus are inhibited by metal-chelating agents such as ethylenediaminetetraacetic acid (EDTA). Optimum activity occurs at neutral pH. Almost all metal-containing proteases are exopeptidases. The acid protease (or aspartic protease) exhibits optimum activity at acid pH. This group of proteases has essential aspartic acid in the active site (Fruton, 1976).

The production of seafood protein hydrolysates may be carried out by employing an autolytic process or an accelerated hydrolysis method involving the use of endogenous or exogenous enzymes, respectively (Mohr, 1977). The endogenous proteases contained in seafood are important because they make a positive contribution to the pleasing aroma, taste, colour and texture of traditional fermented products such as fish sauce, lightly salted fish, dried squid,and caviar (Raksakulkthai *et al.*, 1986; Haard, 1983; Chiou *et al.*, 1989). On the other hand, endogenous enzymes can also have detrimental effects on the quality of raw and cooked fish and shellfish during chill storage or heat processing (Haard, 1994). Post-harvest biochemical changes

caused by endogenous enzymes, including proteases, is the primary cause of quality loss in iced fish and also limits the efficacy of storage strategies, such as modified atmospheres. Furthermore, proteases can be directly responsible for unusual textural defects in seafoods, such as 'belly burst', 'gaping' and 'mushiness' of bony fish and 'tail meat' softening of crustaceans and thus may cause poor initial quality and fillet yields (Martinez and Gildberg, 1988; Honjo *et al.*, 1990; Haard, 1992). Important groups of endogenous fish proteases include those originating from the digestive organs, those present within the muscle cells and enzymes synthesized or secreted in the extracellular matrix. The autolytic hydrolysis lasts from a few days to several months. There are no enzyme costs involved and it is a simple operation. However, prolonged digestion may adversely affect the functional properties of the resultant hydrolysate, and such products are generally used in feed formulations (Shahidi *et al.*, 1995). Therefore, accelerated hydrolysis using commercial proteases is more common, because it offers many advantages over the autolysis, and it also allows the control of hydrolysis and hence manipulation of the properties of the resultant product.

A variety of enzymes from plant, animal and microbial sources have been used to convert seafood wastes and underutilized species to more marketable and acceptable protein hydrolysates (Quaglia and Orban, 1987a; Rebecca *et al.*, 1991; Hoyle and Merritt, 1994; Baek and Cadwallader, 1995; Shahidi *et al.*, 1995; Benjakul and Morrissey, 1997). However, accelerated hydrolysis is generally more complex and the cost of enzyme may influence the economy and commercial viability of the process.

2.4.3 Degree of hydrolysis

The degree of hydrolysis is the percentage number of peptide bonds that are hydrolysed relative to the total number of bonds initially present in the starting protein material. There are various methods of evaluating the degree of hydrolysis (DH) of the peptide bonds. These methods are broadly based on 3 principles; the determination of the amount of nitrogen released during hydrolysis that is soluble in trichloroacetic acid (TCA), the determination of free α-amino groups and the titration of the released carboxyl groups (Silvestre, 1997). To determine the released nitrogen, different methods may be applicable, including Kjeldahl method (AOAC., 1995), spectrophotometric determination in the UV region of the spectrum for peptides with aromatic groups (Pelissier, 1984) or spectrophotometric determination in the visible region after colorimetric reaction such as the Biuret method (Hung *et al.*, 1984).

The formol titration is widely used for the determination of free α-amino groups. The classical Sörensen (1908) method is based on the action of formaldehyde over the amino acids and decreasing the basic character of the α-amino groups. Various compounds are available that are reactive specifically towards the amino groups and are used to determine the DH, such as the ninhydrin (Turgeon *et al.*, 1991), 2,4,6-trinitrobenzene sulphonic acid [TNBS] (Humbert *et al.*, 1990), fluorescamin (Weigele *et al.*, 1972), and orthophthalaldehyde [OPA] (Touati *et al.*, 1992). These methods have different disadvantages such as long analysis time for the ninhydrin technique, interference of reducing sugars and ammonia for the TNBS

method and low stability of the amino derivatives in the use of OPA, amongst others.

The titration of the newly formed carboxyl groups during hydrolysis is known as the pH-stat technique (Aldler-Nissen, 1979). The reaction occurs generally in neutral or slightly alkaline medium which favours the dissociation of the amino groups and the liberation of protons in the reaction medium. Addition of known amounts of alkali to the reaction mixture is continued as the hydrolysis progresses in order to bring the pH of the medium to a desired value; this allows calculation of the degree of hydrolysis. The method is generally used for continuous measurement of the degree of hydrolysis, due to its simplicity, speed and reproducibility. However, the degree of hydrolysis values obtained by this method are relative, and depend on the enzyme and the starting material. Another method which is fairly commonly used to determine the extent of hydrolysis is the measurement of AN/TN ratio, which is the amount of amino nitrogen present in hydrolysate relative to the total amount of nitrogen present in the substrate (Lahl and Braun, 1994). AN/TN ratio is determined by formaldehyde titration (AN) and Kjeldahl method (TN). It is obvious that there is no single, universally used technique for determination of the extent of hydrolysis of proteins and this creates the problem of the inability to compare results of hydrolysis performed by various researchers. A common feature of all techniques is the ability of the experimenter to control the hydrolysis conditions and stop the reaction when the desired degree of hydrolysis is obtained for a particular protein hydrolysate with defined characteristics.

2.4.4 Control of hydrolysis

The ability to control the conditions of the reactions and enzyme activity during hydrolysis has the advantages of producing hydrolysates with narrow molecular weight range such as between 3,000 and 10,000 daltons. The merits of this include lack of bitterness (in most cases), pH-dependent solubility, and enhanced functional properties. Protease activity can be controlled by monitoring the rate of hydrolysis by different methods as described in section 2.4.3.

The extent of hydrolysis can also be controlled by the use of continuous ultra- and hyperfiltration during hydrolysis (Olsen and Adler-Nissen, 1981) or by use of highly specific proteases that carry out limited proteolysis (Whitaker and Puigserver, 1981). Trypsin, acts on arginine or lysine-containing peptide bonds. Furthermore, the plastein reaction has received much attention in the past as controlled method of hydrolysis in order to change the properties of protein system drastically (Fujimaki *et al.*, 1971). In this reaction, a protein is treated with a protease such as papain at neutral pH in order to achieve partial hydrolysis of the protein to fragments of about 10,000 to 20,000 daltons. When the desired degree of hydrolysis is achieved, the protein is concentrated to about 35%, the pH is lowered, and the same protease or another enzyme is used to resynthesize or rearrange a few peptide bonds (Feenney and Whitaker, 1985). The advantages of the plastein reaction are that it can be used to increase the solubility of proteins (when hydrophillic groups are added), change the physical and functional properties of proteins, remove bitter peptides formed with

other proteases, covalently incorporate limiting essential amino acids, remove colour, undesirable flavour and pigments, remove unwanted amino acids and prepare surfactants (Feeney and Whitaker, 1985).

2.4.5 Protein Hydrolysates: Properties and uses in food products

Protein functionality has been broadly defined as "any property of food or food ingredient except its nutritional ones that affects its utilization" (Pour-El, 1981). As protein hydrolysates pose special problems in the formulation of speciality nutritional products, Mahmoud (1994) broadened the definition of functional properties of protein hydrolysates. This definition includes those physicochemical properties that affect the processing, storage stability, flavour quality, and nutritional or biological efficacy of the final product. However, a similar definition is that functional properties of proteins are "those physicochemical properties that govern their performance and behaviour in food systems during preparation, processing, storage and consumption" (Kinsella and Whitehead, 1989). A list of the key physicochemical and related functional properties of protein hydrolysates for nutritional products is given in Table 2.4. Hydrolysis of food proteins is carried for many reasons depending on the source of crude protein to be hydrolysed and the usage of the desired end product. These generally include recovery of protein, improvement of nutritional and/or functional characteristics, retardation of deterioration such as lipid oxidation due to lower lipid content in the hydrolysate, prevention of undesired interactions, and removal of off-

flavours as well as toxic components (Lahl and Grindstaff, 1989; Feeney, 1986; Lahl and Braun, 1994).

Protein hydrolysates possess attractive functional properties as a protein source in human nutrition, both in food products for general and special medical uses. The degree of hydrolysis, being a principal determinant of the properties of protein hydrolysates, is considered the most widely used indicator for comparison among different hydrolysates where the same hydrolysis conditions are applied. However, it is difficult to compare the properties of hydrolysates prepared from different raw materials or methods. Many physicochemical properties that directly affect the functional behaviour of proteins are ultimately related to their amino acid sequence. The amino acid sequence dictates the three-dimensional structure of a protein and thereby its thermodynamic stability, charge distribution pattern on protein surface, symmetric and asymmetric distribution of hydrophillic and hydrophobic patches (cavities) on the surfaces and the topography of the protein surface (Damodaran, 1994). Hence, the hydrolysis process directly influences the functional properties of protein hydrolysates. The functional properties reflect complex interactions that are influenced by the protein composition, its structural conformation and intermolecular association with the other food constituent such as water, carbohydrates and lipids. These interactions are also influenced by the environment in which they occur, and this results in a series of characteristics that enhance the quality and sensory properties of food products (Kinsella, 1982). The nature and content of the amino acids is

Table 2.4 Typical functional properties performed by functional protein in food systems[a].

Functional property	Mode of action	Food system
Solubility	Protein solvation	Beverages
Water absorption and binding	Hydrogen bonding of water; Entrapment of water (no drip)	Meat, Sausages, Breads, Cakes
Viscosity	Thickening; Water binding	Soups, Gravies
Gelation	Protein matrix formation and setting	Meats, Curds, Cheese
Cohesion-adhension	Protein acts as adhensive material	Meat, Sausages, Baked goods, Pasta products
Elasticity	Hydrophobic bonding in gluten; Disulphide links in gels	Meats, Bakery
Emulsification	Formation and stabilisation of fat emulsions	Sausages, Bologna, Soup, Cakes
Fat absorption	Binding of free fat	Meats, Sausages, Doughnuts
Flavour-binding	Adsorption, entrapment, release	Simulated meats, Bakery, etc.
Foaming	Form stable films to entrap gas	Whipped toppings, Chiffon deserts, Angel cakes

[a]Adapted from Kinsella (1982).

crucial to the structure and functionality of protein since these properties are related to the nature of the amino acids either in whole chain or, after enzymatic hydrolysis, in peptides produced from the larger chains. In globular proteins, the hydrophobic amino acids are hidden within the protein molecule, while the hydrophillic side chains predominate on the outside of the molecule. A correlation between hydrophillicity and functional properties has been reported (Bigelow, 1967); the nature of this relationship has also been investigated (MacRitchie, 1978; Graham and Phillips, 1979). In enzymatic hydrolysis, the products will differ from the original protein in the organizational set up.

Of the many actual potential uses of enzymes for modification of proteins and improvement of their functional and nutritional properties, hydrolysis of proteins is the most widely studied (Fox *et al.*, 1982). Functional properties of protein hydrolysates are important, particularly if they are used as ingredients in food products. The main functional properties of protein hydrolysates, which include solubility, emulsification, foaming and viscosity, are common to intact proteins, but differ by process of enzymatic hydrolysis that has occurred, and in most cases enhanced, as compared with those of the original material. Since the functional properties of proteins are dependent on their molecular structure and interaction with the environment, the functional properties of protein hydrolysates depend, to a large extent, on molecular size and degree of hydrolysis (Kester and Richardson, 1984; Adler-Nissen, 1986a; Mahmoud *et al.*, 1992). Moreover, the functional properties of

protein hydrolysates are influenced by the bond specificity of the proteolytic enzymes used, the physical and chemical nature of the intact parent protein and hydrolysis conditions (kester and Richardson, 1984; Gauthier *et al.*, 1993). The cleavage of peptide bonds brings about a complex series of events that alters functionality (Phillips and Beuchat, 1981). The peptides produced by proteolysis have smaller molecular sizes and less secondary structure than proteins and may be expected to have increased solubility near the isoelectric point, decreased viscosity and significant changes in the foaming, gelling and emulsifying properties from those of original proteins. The peptides thus obtained with these characteristics may be useful in various food-processing operations. Peptide size control is necessary if optimum and reproducible changes in functional properties are to be achieved.

Three major changes are known to occur during hydrolysis that have strong influence on the functional properties of the resultant hydrolysate. These changes include (a) an increase in the number of ionizable groups (amino and carboxyl) with the consequent increase in hydrophillicity of the protein (Phillips and Beuchat, 1981) and net charge (Mahmoud *et al.*, 1992); (b) a decrease in molecular size of the polypeptide chain with the associated sharp decrease in antigenicity (Mahmoud *et al.*, 1992) and (c) an alteration of the molecular structure leading to the exposure of embedded hydrophobic groups to the aqueous environment (Phillips and Beuchat, 1981; Kester and Richardson, 1984).

Some functional properties of protein hydrolysates play a more dominant role than others and determine the choice of the hydrolysate for a specific use. For example, the solubility of protein hydrolysates in acidic media may be the most important functional property of hydrolysates used in carbonated drinks. This property would be the main determinant in selecting a given hydrolysate for such application, since it relates directly to the consumer acceptability of the final product (Mahmoud, 1994).

2.4.5.1 Solubility

Solubility is considered the premier functional property because it is usually required for other functional characteristics such as viscosity, gelation, foaming and emulsification. Several parameters are known to affect protein solubility. These include pH, temperature, ionic strength and the presence of other materials capable of binding proteins. The solubility of protein hydrolysates over a wide range of pH, temperature and ionic strength conditions is one of the most important physicochemical and functional properties of hydrolysed proteins. Water is the universal solvent and solubility can give an indication of the effect of heat processing conditions and the potential usage of the protein (Hermansson, 1973; Sathe *et al.*, 1984). Solubility is usually determined by dispersing the protein in water, mixing and adjusting the pH. The mixture is centrifuged and the soluble nitrogen is measured by Kjeldahl method or sometimes by colorimetric procedures and results are expressed

as percentage of total nitrogen solubilized. Most research on protein hydrolysates have been based on the success of hydrolysis measured in terms of their solubilization. Although high solubility is often a prerequisite for good functional properties, it must not be gained at the expense of production of a low average peptide molecular weight (Hall and Ahmad, 1992) and bitter peptides.

Extensive literature support is available on the increased solubility of different proteins through enzymatic hydrolysis (Adler-Nissen and Olsen, 1979; Chobert *et al.*, 1988; Turgeon *et al.* 1992; Shahidi *et al.*, 1995). The type of enzyme used and the conditions of hydrolysis are influenced by the desired product use or the requirements of the food system. In hydrolysis of soy protein isolate, trypsin or Alcalase were more effective than either chymotrypsin or rennet. The hydrolysates prepared using trypsin or Alcalase showed similar solubility at pH 4.5, but at pH 7.0 the former showed better solubility than the latter (Kim *et al.*, 1990). Caseins and caseinates being less soluble at their isoelectric point of 4.6, have limited use in high acid foods and beverages. However, limited hydrolysis of casein with trypsin decreased the average molecular weight to less than 15,000 Da and improved solubility remarkably in the pH range of 4.0 to 5.0 (Chobert *et al.*, 1988). The solubility of protein increases with the degree of hydrolysis, and the degree of hydrolysis increases with incubation time and the amount of enzyme added (Mackie, 1983; Quaglia and Orban, 1987b).

2.4.5.2 Emulsifying properties

Emulsifying properties are also important characteristics usually examined in protein hydrolysates. Emulsions occur in most food systems, and studies on emulsifying agents or materials are consequently given much attention. Emulsifying agents can be defined by their emulsifying capacity (EC), which is the maximum amount of dispersed phase that can be held within the continuous phase by the agent. Emulsion stability describes the ability of the agent to hold the emulsion for a period of time under certain conditions. The emulsification properties of proteins are closely tied to their solubility, hydrophobicity and molecular weight distribution. Alcalase-assisted hydrolysis of sardine proteins showed that emulsifying properties and surface hydrophobicity were reduced when the degree of hydrolysis was increased (Quaglia and Orban, 1990). As the degree of hydrolysis (DH) increased from 5% to 20%, the molecular weight distribution showed an inclination into small molecular weight peptides, lower surface hydrophobicity and poorer emulsifying capacity and stability. The change of pH of the aqueous protein hydrolysate solution in the range of 2 to 8 affected emulsifying properties through effects on solubility of the hydrolysate, and this was noticeable for the low DH samples, although these were better emulsifying agents than the high DH samples at all pH values. Miller and Groninger (1976) working with bromelain-hydrolysed acylated fish myofibrillar proteins showed that acylation tended to improve emulsion properties while enzyme hydrolysis tended to impair them. Rhozyme P-11 was used to hydrolyse fish myofibrillar protein and the

DH in terms of hydrolysis time was related to emulsion capacity and stability (Spinelli *et al.*, 1972). Hydrolysis for varying periods (15 to 90 min) afforded protein hydrolysates with similar emulsion capacities (224 to 231 g oil/g protein), but stability was best at 30 min hydrolysis time, and shorter or longer periods gave poorer stability. Ahmad and Hall (1989) have shown increasing emulsion capacity for protein hydrolysate from cod-filleting waste, with increasing hydrolysis time for trypsin-treated samples, but not for the bromelain-hydrolysed proteins. Samples were hydrolysed for 20 or 60 min and the difference in emulsifying behaviour was associated with the molecular weight distribution of the hydrolysate, solubility/pH and perhaps the presence of suspended proteins. Few emulsifying agents will operate at their maximum capacity in real food systems, thus the true test may be the stability of the systems and it is obvious that solubility/pH relationship are important as in the molecular weight distribution of the protein hydrolysate. Hydrolysis can improve emulsifying properties, but needs to be controlled due to possible formation of bitter-tasting peptides.

2.4.5.3 Fat and water adsorption

The adsorption of oil and water is associated with the physical properties of proteins. As protein hydrolysates are usually very soluble in water, their ability to adsorb oil and water is limited. Miller and Groninger (1976) have shown that acylation of myofibrillar proteins improved their water-holding capacity, while

hydrolysis by bromelain lowered this activity. Fish protein hydrolysates exhibited better fat adsorption than egg albumin or soya bean isolates, and this was attributed to lower particle size in the spray-dried hydrolysates and presence of suspended proteins (Ahmad and Hall, 1989). Water adsorption of the protein hydrolysates was better than egg albumin, but worse than those of soya protein isolates. Moderate fat and water adsorption properties were displayed by protein hydrolysates obtained by Alcalase hydrolysis of seal and capelin proteins (Shahidi *et al.* 1994; Shahidi *et al.*, 1995).

2.4.5.4 Foaming properties

Foams are defined as dispersions of gas bubbles in a continuous liquid or semi-solid phase that contains a soluble surfactant (Dalgleish, 1980). The gas dispersed is usually air or carbon dioxide and the continuous phase is an aqueous solution or suspension containing proteins. The foam expansion and stability of a bromelain-catalysed succinylated fish myofibrillar protein was tested by Groninger and Miller (1975) in model systems and in whole or half substitution for egg albumin in a frozen desert. In the model systems, more extensive hydrolysis tended to give large foam expansion, but the foam was unstable; succinylation to a low to medium level gave better expansion than high-level or unsuccinylated samples. In the use of a similar fish protein hydrolysate, Ostrander *et al.* (1977) tested foaming ability in a whipped gelatin desert where the protein hydrolysate replaced egg albumin at 30, 60 and 100%.

Presence of a residual fishy flavour did not pose any problem as it was masked by other ingredients present in the formulation. Meanwhile, texture, air-cell size and mouthfeel were improved with increasing protein hydrolysate content of the foams compared with the egg albumin foam.

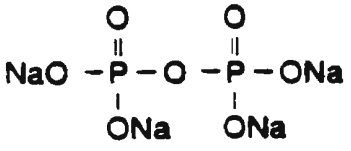
2.4.5.5 Viscosity

Viscosity is the property of a fluid which gives rise to forces that resist the relative movement of adjacent layers in the fluid (Schnepf, 1992). Knowledge about the viscosity of fluids is important in the processing of many foods and will affect the desired texture and appearance of products. Furthermore, viscosity affects other functional properties such as foaming and also gives information on the nature of the proteins involved. Information on the viscosity of protein hydrolysates is very sketchy. Ahmad (1991) has shown an increase in viscosity with concentration at constant temperature and a decrease with increasing temperature at a fixed concentration. The values varied from 1.5 cP for a 5% fish protein hydrolysate solution at 40 °C to 6.6 cP for a 15% hydrolysate solution at 20 °C. The viscosity was very similar to that of egg albumin, but less than that of soya protein isolate. Miller and Groninger (1976) reported that hydrolysis of acylated fish myofibrillar proteins lowered the viscosity which in turn affected foaming activity. The use of a particular protein hydrolysate at levels of 1 to 5% in most food systems may not have a pronounced effect on the viscosity of such products.

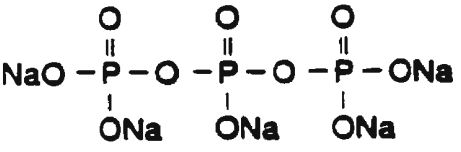
2.4.6 Protein hydrolysates as phosphate alternatives in muscle foods

Phosphates in a variety of forms (monomers, dimeric, trimeric and polymeric phosphates) are commonly incorporated into formulations of processed meat products to improve water- and meat-binding ability, modify textural characteristics, and sometimes prevent formation of off-flavour (Ellinger, 1972; Trout and Schmidt, 1984). Hamm (1970) has summarized the effect of phosphates on the increase in water-binding capacity (and presumably other functional properties such as binding strength) as being due to an increase in pH, an increase in ionic strength, the ability of phosphates to bind meat proteins and the ability of phosphates to dissociate actomyosin into actin and myosin. All of the phosphates used in meat products increased both pH and ionic strength, as these factors are known to increase certain functional properties (Trout and Schmidt, 1986). When used as an additive in meat, phosphates may also attach themselves to the positively charged groups of proteins, while the rest of the molecule can attract water molecules and increase the water holding capacity by acting as a polyanion (Steinhauer, 1983). The interaction or binding of phosphates with proteins through the functional groups of the amino acid components of such proteins makes some amino acids unavailable. This may limit the availability of essential amino acids in some foods where phosphates are used. The structures of commonly used phosphates in foods are given in Figure 2.2. The maximum amount of food-grade phosphates permitted for incorporation into meat products is 0.5% (USDA, 1982).

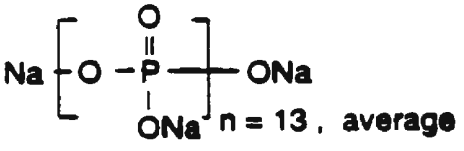
Figure 2.2 Structures of some commonly used phosphates in food.



Tetrasodium pyrophosphate (TSPP)



Sodium tripolyphosphate (STPP)



Sodium hexametaphosphate (SHMP)

The improvement of water-binding capacity and other functional properties of muscle proteins may be achieved by addition of protein hydrolysates (Schnepf, 1992). Addition of protein hydrolysates renders water retention in meat systems and, in this regard, may be used as phosphate alternatives. Seal protein hydrolysates have exhibited enhanced water binding capacity when used in a meat system as indicated by their ability to reduce the amount of drip volume during heat processing (Shahidi and Synowiecki, 1997).

2.4.7 Taste of protein hydrolysates

The taste of protein hydrolysates depends on the primary structure of the original protein. In addition to the bitterness brought about by certain peptides released during hydrolysis, there are other sweet, sour, brothy or beefy tastes associated with amino acid and peptide residues (Hamada, 1994). Free amino acids which are usually in high levels in hydrolysates as compared with their original raw material contribute sweet, bitter, sour and umami characteristics to foods (Solms, 1969). The sweet taste of lobster and crab is due to glycine and the characteristic taste of sea urchin is due to methionine (Pan and Kuo, 1994). The bitter taste produced during the storage of cheese and in the fermentation of the traditional Japanese food "miso" and "nato" has been shown to be due to peptides resulting from the hydrolysis of proteins. Glycine and L-alanine show strong sweetness and most L-amino acids exhibit bitterness, whereas all D-amino acids are sweet (Nashimura and

Kato, 1988). Glutamic acid residue in proteins is not a flavour enhancer, but glutamate bound to a peptide may have flavour-enhancing properties as is also the case for monosodium glutamate. Ariyoshi (1976) synthesized various peptides and examined their sweet taste intensities. He demonstrated that a relationship existed between their sweet taste intensity and structure of test materials. Studies on some tetrapeptides and their derivatives were reported by Ariyoshi (1985) where he concluded that the longer the peptide chain, the more difficult the binding of the peptide to receptor pocket and hence several tetrapeptides generally elicit bitter taste.

A slight amount of bitterness is associated with some foods and beverages, such as beer, bitter melon, coffee and grapefruit, but in most foods, bitterness is not desirable. The hydrophobic amino acids, L-Phe, L-Tyr, L-Trp, L-Leu, L-Val and L-Ile elicit bitter taste (Nishimura and Kato, 1988). This observed characteristic indicates that bitterness of peptides may be caused by the hydrophobic property of their amino acid side chains.

Protein hydrolysates from enzyme-hydrolysed raw materials have been widely used as ingredients in a variety of foods. However, one feature which hinders their wider acceptance in some foods is the presence of bitter peptides, attributed to their hydrophobic amino acid content (Matoba and Hata, 1972; Ney, 1979) and arising as natural degradation products of the proteolytic reaction. Many bitter peptides have been isolated from products of enzymatic hydrolysis of casein, soybean protein and cheese as well as other foods, and their structures determined. The bitterness of

peptides can be predicted by calculation of the Q value of a peptide, which depends on the amino acid composition. The Q value represents the average hydrophobicity of a peptide and is calculated as the average free energy of transfer of the amino acid side chains from ethanol to water, divided by the number of amino acid residues as described by Ney (1971). When the Q value is greater than 1.4 kcal mol^{-1}, bitterness is likely to occur (Ney, 1972). To avoid bitterness in this case, the relative molecular mass of a peptide must be as high as possible and more than 6000 daltons when considering hydrolysates of casein, potato and wheat gluten (Ney, 1978). Gelatin hydrolysates, however, were not bitter at any molecular weight level. The results of enzymatic hydrolysis of casein show that for larger peptides, neither hydrophobicity nor size alone is responsible for bitterness, but some conformational parameters may be of greater importance. It has been further concluded that only a part of the peptide structure is responsible for contact with the taste receptor (Ishibashi *et al.*, 1988; Bumberger and Belitz, 1993). A good correlation existed between the molecular weight distribution of peptides and the bitter taste of protein hydrolysates (Ney, 1979). The structure of bitter peptides isolated from soy protein (Fujimaki *et al.*, 1968), zein (Wieser and Belitz, 1975) and casein (Matoba *et al.*, 1970; Minamiura *et al.*, 1972; Clegg *et al.*, 1974) have been identified (Table 2.5).

The bitter taste elicited in protein hydrolysates is caused by small hydrophobic peptides which are not present as such in the original protein source. The globular structure of the native protein containing the majority of the hydrophobic side chains

Table 2.5 Bitter Peptides isolated from different protein sources[a].

Protein source	Enzyme	Peptides
Soy protein[1]	Pepsin	Gly-Leu; Leu-Phe; Leu-Lys; Arg-leu; Arg-Leu-Leu; Ser-Lys-Gly-Leu.
Zein[2]	Pepsin	Ala-Ile-Ala; Ala-Ala-Leu; Gly-Ala-Leu; Leu-Gln-Leu-Leu-Glu-Leu; Leu-Val-Leu; Leu-Pro-Phe-Asn-Gln-Leu-Leu-Pro-Phe-Ser-Gln-Leu.
Casein[3]	Papain	Ala-Gln-Thr-Gln-ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln.
Casein[4]	Trypsin	Gly-Pro-Phe-Pro-Val-Ile; Phe-Phe-Val-Ala-Pro-Pro-Glu-Val-Phe-Gly-Lys; Phe-Ala-Leu-Pro-Glu-Tyr-Leu-Lys.
Casein[5]	Bacterial protease	Arg-Gly-Pro-Pro-Phe-Ile-Val(BP-Ia); Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile-Asn-His; Cyclo(Leu-Trp-Leu-Trp).

[a]References: [1]Fujimaki *et al.* (1968); [2]Wieser and Belitz (1975); [3]Clegg *et al.* (1974); [4]Matoba *et al.* (1970); [5]Minamiura *et al.* (1972).

is folded into the interior of the molecular structure and hence cannot interact with the taste buds (Adler-Nissen and Olsen, 1979). During hydrolysis, the long polypeptide chains are degraded to smaller peptides, but may be sufficiently long to maintain a cluster of configurations and the hydrophobic side-chains remain hindered. As the hydrolysis proceeds, the shorter chain peptides formed are unable to maintain their original configurations and the hydrophobic side-chains become exposed, thus resulting in increased bitterness of products. When proteins are extensively hydrolysed, smaller bitter peptides which have terminal hydrophobic groups are degraded to peptides with less hydrophobic end groups and release of free amino acids with associated decrease in degree of bitterness (Adler-Nissen and Olsen,1979).

2.4.7.1 Methods of controlling bitter taste of peptides

A variety of methods are available for controlling the bitter taste of protein hydrolysates, and these include masking with other substances, removal of hydrophobic peptides by using hydrophobic materials and control of proteolysis by choosing appropriate enzymes for hydrolysis. Masking effect on bitter peptides may be achieved by different means, such as use of glycine, a sweet-tasting amino acid which has been applied to mask the bitterness of chicken protein hydrolysate (Stanley, 1981). Removal of bitter peptides from protein hydrolysates has been carried out using a variety of hydrophobic materials e.g. talc (Chersi and Zito, 1976), phenolic formaldehyde resin (Roland *et al.*, 1978), activated carbon (Shahidi *et al.*, 1995), glass

material and hexylepoxy cellulose (Helbig *et al.*, 1980) and recently octadecylsiloxane (C18) adsorption columns (Lin *et al.*, 1997). Other methods used to control bitter taste in hydrolysates include controlling the degree of hydrolysis, plastein reaction (Synowiecki *et al.*, 1996), and the most widely used is the release of hydrophobic amino acids from bitter peptides by exopeptidases (Umetsu *et al.*, 1983) and aminopeptidases (Matsuoka *et al.*, 1991; Tan *et al.*, 1993). The choice of appropriate enzyme plays an important role in the formation of bitter-tasting peptides (Adler-Nissen, 1986a). Endopeptidases hydrolyse protein molecules from within the peptide chain and exopeptidases specifically cleave terminal amino acids from peptides. Endopeptidases when used alone to hydrolyse peptide linkages within the protein molecules generally generate bitter peptides. However, it has been reported that hydrophobic amino acids at terminal positions of peptides result in more bitterness than in endo positions (Adler-Nissen, 1986a). Hence, a protein hydrolysate made with Alcalase or proteases with similar specificity will produce less bitter-tasting peptides than a hydrolysate made with endopeptidases. Hevia *et al.* (1976) found that casein treated with papain and ficin endopeptidases produced more bitter peptides than Pronase, an exopeptidase, which produced a brothy tasting hydrolysate. The use of both exopeptidase and endopeptidase in hydrolysis of casein to produce a relatively bitter-free tasting hydrolysate was reported by Clegg and McMillan (1974). Over 50% free amino acids was present in such hydrolysate due to extensive hydrolysis by both enzyme types.

Different types of chromatographic techniques have been developed for the separation of proteins and their hydrolysates. Some of these techniques which have been employed to separate different peptides, based on their hydrophobicity, have aided in the isolation and identification of bitter-tasting peptides. Reverse phase high performance liquid chromatography was used to isolate the bitter peptide fractions present in a tryptic casein hydrolysate (Minagawa *et al.*, 1989). Kukman *et al.* (1995) isolated small hydrophobic bitter peptides and determined their molecular mass distribution in soybean protein hydrolysates using gel permeation HPLC, gel chromatography and reverse phase HPLC. The low molecular weight peptides extracted with 2-butanol from hydrolysates had a higher bitterness intensity. The most bitter low-molecular mass fractions of peptides were reported to be those with a molecular weight lower than 1000 Da and were obtained after gel permeation chromatography on a Sephadex G-25 column. The small hydrophobic peptides were slightly bitter in addition to having a sour, burning or salty taste (Kukman *et al.*, 1995).

It has been reported that incorporation of glutamic acid in bitter protein hydrolysates by the plastein reaction eliminated the bitterness because of the masking effect of glutamic acid residues in new acidic oligopeptides (Tamura *et al.*, 1990). Thus, it may be possible to use deaminated protein hydrolysates for flavour enhancement. It may be beneficial, however, to develop a protease that is tailored for

the production of flavour enhancers by recombinant DNA and site-directed mutagenesis techniques (Kukman, 1995). The protease(s) should be tailored to produce protein hydrolysates that lack bitterness and after modification such as deamination may impart meaty or other flavours when added to meat products (sausages, burgers, etc.), cheeses or texturized vegetable proteins.

2.4.8 Separation techniques for peptides of protein hydrolysates

Mixtures of peptides may be separated by exploiting the characteristic properties of functional groups present in the amino acid subunits of the peptides. Usually such separations are performed using thin-layer chromatography (TLC), ion-exchange chromatography, high performance liquid chromatography (HPLC) and gel electrophoresis. The low resolving ability of any one technique usually requires a combination of two or more techniques to obtain highly resolved peptides (Kratzin and Yang, 1981). Most studies on peptide separation have been carried out using casein hydrolysate. This protein has commonly been used because it is edible, its composition is known and it is usually used as a substrate for the digestion test by proteinases (Adachi *et al.*, 1991). Moreover, to visualize the peptide fragments, colour producing agents such as ninhydrin, o-phthalaldehyde or fluorescamin have been used (Moore and Stein, 1954; Roth, 1971; Weigele *et al.*, 1972; Benson and Hare, 1975). However, these compounds even in low concentrations result in loss of some peptide

material (Kratzin and Yang, 1981).

Gel filtration or cation exchange columns in weak phosphoric acid solutions may be successfully employed for some separations (Chin and Wold, 1977). While this permits UV detection at 206 nm and higher, large peptides are frequently insoluble in phosphoric acid, and the solvent is not totally volatile and may require cumbersome desalting procedures (Mahoney and Hemodson, 1980). The use of HPLC to isolate peptides has received increasing attention from protein chemists in the past two decades. There has been considerable success in purifying native proteins and peptides obtained from enzymatic hydrolysis. The successful isolation of large peptides for the sequence determination of rabbit liver cytochrome b_5 has been reported (Takagaki *et al.*, 1980). The authors used reversed phase HPLC on C-18 column with a mobile phase consisting of formic acid, water and ethanol. Separation of short- and medium-chain peptides, using 5 μm ODS column, indicates that the retention time of a peptide in the potassium phosphate system employed is determined by its polarity (Mönch and Dehnen, 1977). Moreover, the polarity of the C-terminal amino acid of dipeptides and the overall level of polar amino acid content and the length of the peptide chain influences its retention on the stationary phase of the column. The separation and identification of peptides and amino acids of hydrolysed proteins have been studied using different methods in order to obtain a better knowledge, and hence understanding, of the characteristics of protein hydrolysates.

Among the methods that have been used for this purpose, high performance liquid chromatography (HPLC), especially in the reverse phase mode (RP-HPLC), may be noted. This procedure is efficient for separation of peptides from protein hydrolysates based on their hydrophillic and hydrophobic characteristics (Lemieux *et al.*, 1991).

Different experimental conditions of RP-HPLC for separating the peptides from trypsic hydrolysates of casein, from β-lactoglobulin A, and from human growth hormone (Kalgahati and Horváth, 1987; Maa and Horváth, 1988) have been employed. The RP-HPLC was also used to monitor the peptides produced at different stages of hydrolysis of whey protein using commercial proteinases (Perea *et al.*, 1993). Imbert and Nicholas (1993) proposed a RP-HPLC technique for separating the two peptides obtained from *k*-casein hydrolysis by rennet, A and B caseinomicropeptides, that enabled the identification of whey protein adulterated milk or milk powder. The RP-HPLC technique has also proven useful in the separation of bioactive peptides from protein hydrolysate (Bouhallab *et al.*, 1993). The elution profiles on a RP-HPLC column was also examined for antioxidative peptides from soybean protein (β-conglycinin) hydrolysate (Chen *et al.*, 1995).

In spite of the high resolution capacity of RP-HPLC, the procedure allows only a partial characterization of protein hydrolysates. The separation criteria employed in these phases, which are based on hydrophobicity or charge of peptides, are not good for characterising protein hydrolysates from a nutritional point of view, where peptide

size is the essential quality parameter. The size-exclusion chromatography (SEC) or gel permeation chromatography, essentially in high performance liquid chromatography mode (SE-HPLC), is one of the desired techniques allowing the study of chromatographic profiles of protein hydrolysates. The peptide retention on a reverse-phase column depends on the amino acid composition, ionization state, charge and hydrophobicity, while the molecular volume is the determining factor for their separation on a size-exclusion support; the peptides fractionated by SE-HPLC may then be isolated and purified by RP-HPLC in order to obtain more information about peptide and amino acid composition of protein hydrolysates. This process was used by Lemieux *et al.* (1991) for analyzing casein hydrolysates. Different materials have been employed as support for separating peptides on the basis of their size. Some of these column materials include Sephadex G-25, Sephadex G-10 and Biogel P-2 (Landry *et al.*, 1988; Iliev and Tchorbanov, 1992; Zhang *et al.*, 1992; Amarowicz and Shahidi, 1997). However, Pellerin *et al.* (1985) used different Sephadex types and varied the molecular size fractionation, ranging from G-10 to G-200, to study the relative peptide and amino acid composition of some acidic and enzymatic protein hydrolysates of milk proteins.

The ligand exchange chromatography (LEC) involving the complex formation with metallic ions, mainly copper (II), is another technique used for separating peptides and amino acids of protein hydrolysates. Verneuil *et al.* (1990) used LE-

HPLC to fractionate a bovine serum albumin hydrolysate into its amino acids, dipeptides, tripeptides and larger size peptides using silica gel bonded with copper (II). Another separation technique which may be used to study the profile of protein hydrolysate is the capillary electrophoresis (CE) which has the advantage of high sensitivity and resolution. CE coupled with RP-HPLC was used to analyze a tryptic myoglobin hydrolysate (Castangola *et al.*, 1991). These two techniques gave complimentary results because the separation on a RP-HPLC column depends on polarity, while that on CE is based on the peptide charge.

The efficiency of five chromatographic systems for separation of commercial casein hydrolysates was tested by Lemieux and Amiot (1990) using size exclusion or gel filtration, reversed phase and ion-exchange HPLC, as well as affinity chromatography. HPLC systems showed several advantages over other chromatographic methods, like SEC and metal affinity chromatography. Among these advantages are the short time required for analysis and high resolution. However, the best system for determination of molecular weight distribution of hydrolysates was the SE-HPLC. The various separation techniques have allowed isolation of certain peptides with specific physical and chemical characteristics which enabled their properties to be studied.

2.5 Lipid autoxidation - General considerations

Lipid oxidation in is a major cause of food quality deterioration, resulting in changes in favour, colour, texture and nutritive value during processing and storage. Moreover, the presence of lipid oxidation products such as lipid peroxides, aldehydes and oxidized cholesterol derivatives in foods pose a health risk because these compounds are potentially toxic (Sevanian and Peterson, 1986; Chan and Decker, 1994). Lipid autoxidation is the deterioration of polyunsaturated fatty acids which occurs via a free radical-mediated phenomenon involving a chain reaction mechanism. It leads to the formation of unstable primary products and finally stable secondary products. Autoxidation of unsaturated lipids is, in general, classified into three separate reaction phases: initiation, propagation and termination.

The initiation step, which usually results in the formation of free radicals of the unsaturated fatty acids, occurs with or without the interaction of oxygen, in the presence of catalysts such as heat, light, or transition metal ions. The next phase basically involves the continuation and acceleration of the chain reaction which had begun in the initiation step. The peroxy radical can abstract a hydrogen atom from an adjacent fatty acid to produce a free radical. The free radical produced ($R^{\bullet}$) can then oxygenate, to form a peroxy radical, which may subsequently attack another lipid molecule or the starting molecule to remove a hydrogen atom. In this way a hydroperoxide is formed and another free radical is produced (Eq. 3). Thus, the oxidation continues in a chain reaction (propagation phase). The hydroperoxides are

Initiation:

$$RH \rightarrow R^{\bullet} + H^{\bullet} \quad (1)$$

Propagation:

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (2)$$

$$ROO + RH \rightarrow ROOH + R^{\bullet} \quad (3)$$

Termination:

$$R^{\bullet} + R^{\bullet} \rightarrow RR \quad (4)$$

$$R^{\bullet} + O_2 \rightarrow ROOR \quad (5)$$

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2 \quad (6)$$

unstable, hence they degrade easily to give two radicals (either an alkoxide and a hydroxyl) or a peroxy free radical, hydroxyl free radical and water. This leads to proliferation of free radicals which may go back to aid the propagation step, hence it proceeds autocatalytically (Hamilton *et al.*, 1997). Hydroperoxides (ROOH) may also decompose to produce alcohols, aldehydes, acids, ketones and less reactive substances. The termination stage of this chain reaction occurs by the reaction of two radical species to form non-radical molecules (Loliger and Wille, 1993; Cuppett *et al.*, 1997). This step completes one cycle of lipid autoxidation, however, there can be reinitiation and the cycle may repeat itself. This may continue indefinitely or until oxidizable

lipid substrates are depleted. The formation of free radicals may occur in biological or food systems under appropriate conditions, such as presence of initiators, including light, metal ions, pigments, etc. Lipid oxidation, besides causing rancid odour in food, also initiates other changes that invariably influence or affect various quality aspects. Nutritional quality may be impaired by losses in essential fatty acids, essential amino acids, proteins and vitamins. Various oxidized products generated during lipid oxidation may be acutely toxic or mutagenic (Das, 1993). In addition, the flavour, texture and colour of meat and plant materials may be affected by the bleaching of carotenoids, myoglobin, anthocyanins and chlorophyll by the formed peroxides (Finley and Given, 1986). The formation of free radicals in biological systems can occur through either enzymatic or non-enzymatic processes and their formation can occur under normal and pathological conditions. Several biochemical pathways can lead to the formation of many reactive oxygen species (ROS) intermediates such as superoxide , hydrogen peroxide and hydroxyl radical, any or all of which could lead to the formation of free radicals and/or lipid hydroperoxides. Excessive generation of free radicals would occur when an imbalance occurs between rate of their formation and the several protective mechanisms available for their removal.

2.5.1 Food antioxidants

Food antioxidants are substances which when present in foods at low concentrations, as compared with those of oxidizable substrates, markedly delay or

prevent the oxidation of such substrates (Halliwell *et al.*, 1995). Food manufacturers have used food grade antioxidants not only to extend shelf life of products, but to reduce wastage of raw material as well as nutritional loss, and to widen the range of fats that can be used in specific products (Coppen, 1983). Antioxidants have also been of interest to biochemists and health professionals because they may help the body against damage by reactive oxygen species (ROS) and from degenerative diseases (Shahidi, 1997). The antioxidants used in food may be grouped as synthetic or natural, depending on their source and mode of production.

2.5.1.1 Antioxidant classification

Food antioxidants can be broadly classified into primary and secondary groups on the basis of their mechanism of action. Some antioxidants exhibit more than one mechanism of activity and are often referred to as multiple-function antioxidants (Reische *et al.*, 1998). Chemical modes of action vary greatly as these substances are able to function at all stages of free radical reaction.

2.5.1.1.1 Primary antioxidants

Primary antioxidants, type 1 or chain-breaking, are free radical acceptors that delay or inhibit the initiation step or interrupt the propagation step of autoxidation. They react with high-energy lipid radicals to convert them to thermodynamically more

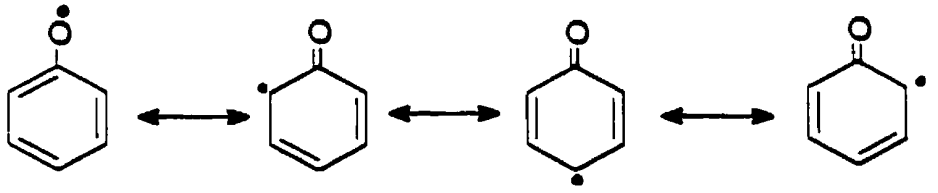
stable products (Shahidi and Wanasundara, 1992). Primary antioxidants donate hydrogen atoms to the lipid radicals and produce lipid derivatives and antioxidant radicals ($A^{\bullet}$) that are more stable and less readily available to further promote autoxidation. As hydrogen donors, primary antioxidants have higher affinities for peroxy radicals than lipids (Porter, 1980). Therefore, peroxy free radicals formed during the propagation phase of autoxidation are scavenged by primary antioxidants (Eq. 7). Antioxidant may also interact directly with lipid radicals (Eq. 8).

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet} \quad (7)$$

$$R^{\bullet} + AH \rightarrow RH + A^{\bullet} \quad (8)$$

The radical produced by hydrogen abstraction has a very low reactivity with lipids and hence reduced rate of propagation, since reaction of antioxidant radicals with oxygen or lipids is very slow. The antioxidant radical is stabilized by delocalization of the unpaired electron around a phenolic ring to form a stable resonance hybrid (Figure 2.3). The most commonly used primary antioxidants in food are synthetic compounds, including phenolic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tertiary-butylhydroquinone (TBHQ). However, some natural components of food also act as primary antioxidants and among them are those which are phenolic in nature.

Figure 2.3 Resonance stabilization of phenoxyl radical.



2.5.1.1.2 Secondary antioxidants

Secondary, preventive, or type 2 antioxidants act through different mechanisms. The antioxidants retard the rate of oxidation by several different actions, but they do not convert free radicals to more stable products. Secondary antioxidants can chelate prooxidant metals and deactivate them, replenish hydrogen to primary antioxidants, decompose hydroperoxides to nonradical species, deactivate singlet oxygen, absorb ultraviolet radiation or act as oxygen scavengers (Reische *et al.*, 1998). These antioxidants are often referred to as synergists because they promote the antioxidant activity of the primary antioxidants. Citric acid, tartaric acid and lecithin are examples of synergists.

The chelators are types of secondary antioxidants which complex heavy metals with two or more valence states (Fe, Cu, Mn, Zn, etc.) which promote oxidation by acting as catalysts of free radical reactions. Chelation of metals by certain compounds decreases their prooxidant effect by reducing redox potentials and by stabilizing the oxidized form of the metal. Chelating compounds may also sterically hinder formation of the metal hydroperoxide complex. Examples of chelators are citric acid, phosphoric acid (and its polyphosphate derivatives), and ethylenediaminetetraacetic acid (EDTA).

Ascorbic acid, ascorbyl palmitate, erythorbic acid, sodium erythorbate and sulphites are secondary antioxidants which prevent oxidation by scavenging oxygen and acting as reductants. Finally, the singlet oxygen quenchers, which include

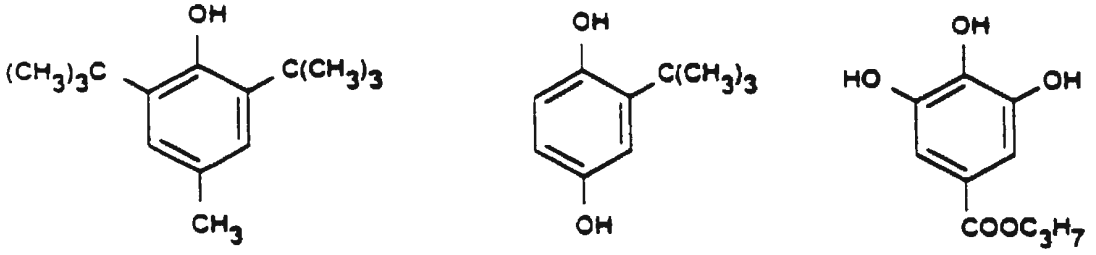
carotenoids such as β-carotene and lycopene, act by depleting singlet oxygen of its excess energy and dissipating the energy in the form of heat.

2.5.1.2 Synthetic antioxidants

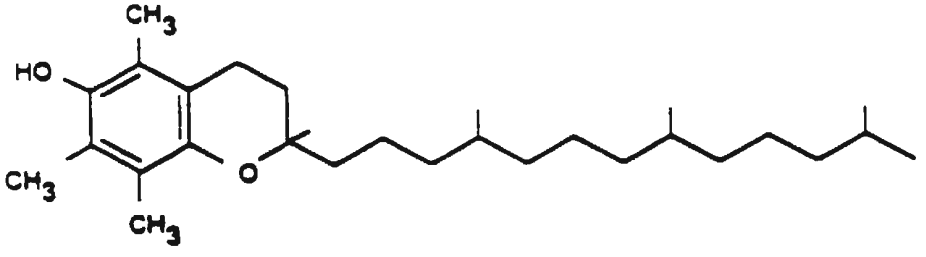
Antioxidants mostly used in foods are synthetic and these include BHA, BHT, PG which are permitted for use in food in Canada and TBHQ; the latter also is allowed for use in the United States. Antioxidants are known to act at different levels in the oxidative sequence involving lipid molecules. They act by decreasing oxygen concentration, intercepting singlet oxygen, preventing first-chain initiation by scavenging initial radicals such as hydroxyl radicals, binding metal ion catalysts, decomposing products to non-radical compounds, and chain breaking in order to prevent continued hydrogen abstraction from substrates (Shahidi, 1997). In general, the free radical chain reactions within a substrate can be inhibited either by adding substrates that would retard the formation of free radicals (preventive antioxidants), or by introducing substances that would compete for the existing radicals and remove them from the reaction (chain-breaking antioxidants). Most research in the field of lipid oxidation prevention is concerned with the second mechanism, designing or isolating chemicals or substances which when added in small quantities to the substrate react rapidly with the free radical intermediates of an autoxidation chain and stop it from progressing. The chemical structures of some synthetic antioxidants, including α-tocopherol (may also be regarded as natural antioxidant) are given in Figure 2.4.

Figure 2.4 **Structures of some synthetic food antioxidants.**

BHA (two isomers)



BHT TBHQ PG



α-Tocopherol

2.5.1.3 Natural antioxidants

Some naturally-occurring substances, including those found in animals and plants, possess antioxidant activity (Chan and Decker, 1994). The mechanism by which these antioxidants control autoxidation and rancidity development in foods may be different. The most important commercial natural antioxidants are ascorbic acid and tocopherols. Other natural antioxidants include phenolic and polyphenolic compounds, carotenoids, flavonoids, enzymes, protein, protein hydrolysates, amino acids, Maillard reaction products, phospholipids and sterols. Naturally-occurring antioxidants have been mostly identified in plant sources including vegetable extracts. Among the most popular plant natural antioxidants are those from rosemary, sesame sage, oat, amongst others (Schuler, 1990).

Natural antioxidants allow food manufacturers to produce stable products with labels that entail all-natural ingredients which are attractive to the consumers because of concerns of possible adverse effects of synthetic antioxidants. However, these products may have several drawbacks, including high level of usage, undesirable flavour or colour and lack of stability due to low antioxidant efficiency. Nonetheless, the merit of using natural antioxidants tend to outweigh the above drawbacks as natural antioxidants are still the preferred food ingredients for consumers because of their desired and beneficial health effects.

2.5.1.2 Endogenous antioxidative compounds of animals - General considerations

Natural antioxidants in foods may be endogenous or formed as a result of reactions during processing, and food additives isolated from natural sources (Pratt and Hudson, 1990). Muscle foods are susceptible to lipid oxidation reactions, especially under conditions such as grinding, which incorporates oxygen into the muscle, and releases hemoprotein into contact with lipid components of the muscle; then cooking, which releases protein-bound iron into the intracellular pool (Chan and Decker, 1994). This combination of lipid oxidation catalysts, highly unsaturated membrane systems, and periods of high oxygen tension leads to oxidative reactions in muscle that occur initially at membrane level (Wilson *et al.*, 1976). To prevent or retard oxidative reactions, several endogenous antioxidant systems are found in muscle tissues. These endogenous antioxidants include tocopherol and other phenolic compounds, histidine-containing dipeptides, antioxidant enzymes such as glutathione peroxidase, superoxide dismutase and catalase. The antioxidant systems are effective in inhibiting lipid oxidation catalysts and preventing the formation of free radicals. Because these antioxidants are naturally present in skeletal muscles, they are able to inhibit lipid oxidation both in the living tissue and in the muscle foods.

2.5.1.2.1 Endogenous antioxidants of shellfish

The earliest indication of possible presence of antioxidant components in shellfish was reported by Babbitt *et al.* (1974). They incorporated shrimp (*Pandalus*

jordani) meat in a fish-shrimp portion and found an increased shelf-life of machine-separated fish muscle which was attributed to the presence of shrimp. In another study, decreased rate of peroxide and malonaldehyde formation, over a 12-week storage period under frozen conditions, was reported for rockfish-shrimp portions as compared with only fish portions. In general, a decreased malonaldehyde and peroxide levels was proportional to the levels of shrimp substituted in this portion. The ethanolic extract of shrimp muscle exhibited strong antioxidant activity by delaying the bleaching of β-carotene, and a partial characterization of ethanol-extracted component recognised the compound(s) to be phenolic in nature (Pasquel and Babbitt, 1991). These authors, however, speculated that the antioxidant compound may be a hydroxylated derivative of an aromatic amino acid. Recently, Li *et al.* (1996) extracted antioxidant components from shrimp waste using various organic solvents. As in the case of the earlier study by Pasquel and Babbitt (1991), ethanol was most effective in extracting the antioxidants indicating the polar nature of such compounds. The antioxidant activity of the crude and purified sample was not affected by heating at 100 °C for up to 4 h or during storage at 4 °C for 30 days (Li *et al.*, 1996).

2.5.1.3 Antioxidant properties of amino acids, peptides, proteins and hydrolysates

Amino acids in the free form or as components of peptides are present in both plant and animal tissues as a result of incomplete protein synthesis or due to partial

degradation of proteins. In the mammalian system, such free amino acids form the physiological amino acid pool. In food systems, apart from their role as flavour precursors, free amino acids and peptides have been reported to exhibit an antioxidative effect under certain experimental conditions (Marcuse, 1960, 1962).

Proteins, peptides and free amino acids decrease the rate of autoxidation and the hydroperoxide content of lipid-containing foods. Amino acids and peptides exhibit metal-chelating properties in foods and are found in abundance in protein hydrolysates. In a suitable environment, such as bulk oil and in a linoleic acid model system, many amino acids exhibit antioxidative effects (Ahmad *et al.*, 1983). However, the pro-oxidant effect of some amino acids under certain conditions such as high concentration of amino acids and the absence of metals, when present in an aqueous media have been reported (Farag *et al.*, 1978a,b).

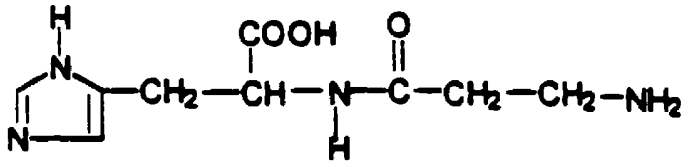
Certain amino acids, peptides and proteins act as antioxidants under suitable conditions. Marcuse (1961) reported the antioxidative effects of several amino acids in aqueous solutions of linoleate and concluded that histidine, alanine, methionine and lysine exhibited antioxidative activity. In the same study these amino acids were pro-oxidative at high concentrations. The pH of the medium and the presence of other antioxidants also influenced the antioxidative effects of the amino acids. Amino acids are effective synergists in combination with phenolic antioxidants, acting both as chelators of heavy metals and as promoters of decomposition of hydroperoxides, which are otherwise a source of free radicals (Pokorny, 1991). In the presence of

amino acids, copper complexes are formed and translocated to the aqueous phase and rendered innocuous as catalysts of lipid oxidation (Marcuse, 1962).

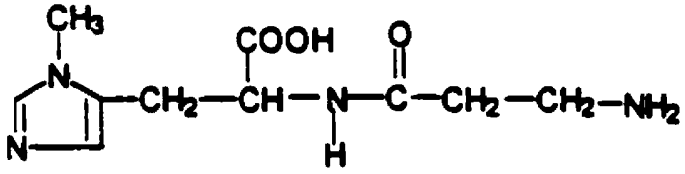
Dipeptides, especially those having branched-chain amino acids, showed considerably higher antioxidant activities than others devoid of branched chain amino acids in an oil system (Kawashima *et al.*, 1979). These researchers further reported that the type of terminal amino acid in a dipeptide also influences the antioxidant activity observed, as N-terminal branched chain amino acid was preferred over a C-terminal one for antioxidant activity. The effectiveness of these peptides as antioxidants was in the order Leu-Gly > Leu-Sar > Ile-Gly > Leu-Ile > Leu-Met > Val-Gly.

Other peptides have been found to exhibit antioxidant activity. Carnosine (β-alanyl-L-histidine), anserine (β-alanyl-L-1-methylhistidine) and ophidine (β-alanyl-L-methylhistidine) are natural imidazole-containing compounds (Figure 2.5) found in non-protein fraction of mammalian tissues. The dipeptides carnosine and anserine are found in the concentrations of 1 to 25 mM in skeletal muscles of most vertebrates where they exhibit excellent buffering capacity at physiological pH values (Harris *et al.*, 1990), which explains why higher concentrations of these compounds are found in white muscle fibres in which high levels of anaerobic metabolism are common. These peptides are capable of inhibiting iron-catalyzed lipid oxidation while their individual amino acids were ineffective (Boldyrev *et al.*, 1988; Decker and Faraji, 1990). These peptides have also been reported to play a role in the regeneration of

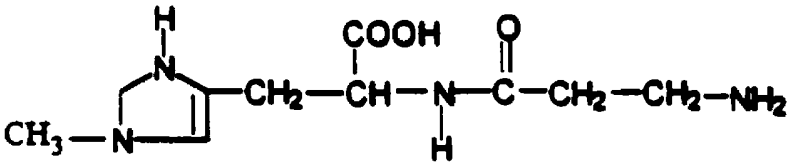
Figure 2.5 **Structures of some imidazole dipeptides.**



Carnosine



Anserine



Ophidine

α-tocopherol (Boldyrev *et al.*, 1988), suggesting that they could act synergistically with α-tocopherol to inhibit lipid oxidation. The mechanism of action of carnosine and anserine as antioxidants has been studied over the past few years and mechanisms have been postulated which support metal chelation and free radical-scavenging effect of these dipeptides. Whereas carnosine and anserine exhibit antioxidant activity, their constituent amino acids, β-alanine and histidine are not as effective in inhibiting oxidation in phosphatidylcholine liposomes and sarcoplasmic reticulum when used individually or in combination (Chan *et al.*, 1994; Decker *et al.*, 1992). This suggests that the peptide linkage between β-alanine and histidine is involved in the antioxidant activity of histidine-containing dipeptides.

Electron paramagnetic resonance (EPR) studies have shown that histidine and histidine-containing dipeptides such as glycine-histidine, carnosine and homocarnosine possess hydroxyl radical-quenching activity, whereas non-histidine dipeptides such as β-alanine-glycine and β-alanine-alanine do not quench hydroxyl radicals (Chan *et al.*, 1994). This further suggests that the histidine group of carnosine is responsible for its hydroxy radical-scavenging activity. The importance of the presence of histidine on the antioxidant activity of peptides in other studies has been reported (Chen *et al.*, 1995). The antioxidant activity of histidine-containing dipeptides was in the order of carnosine > homocarnosine > glycine-histidine (Chan *et al.*, 1994).

Six antioxidative peptide fragments from the digest of a soybean protein, β-conglycinin, have been isolated (Table 2.6). The peptides were composed of 5-16

Table 2.6 Amino acid sequences of six antioxidative peptides derived from hydrolysis of β-conglycinin[a].

1. Val-Asn-Pro-His-Asp-His-Gln-Asn
2. Leu-Val-Asn-Pro-His-Asp-His-Gln-Asn
3. Leu-Leu-Pro-His-His
4. Leu-Leu-Pro-His-His-Ala-Asp-Ala-Asp-Tyr
5. Val-Ile-Pro-Ala-Gly-Tyr-Pro
6. Leu-Gln-Ser-Gly-Asp-Ala-Leu-Arg-Val-Pro-Ser-Gly-Thr-Thr-Tyr-Tyr

[a]Adapted from Chen *et al.*, 1995.

amino acid residues, including hydrophobic amino acids, Val or Leu, at the N-terminal positions and Pro, His, or Tyr in the sequences (Chen *et al.*, 1995). In a further study, the smallest peptide, Leu-Leu-Pro-His-His, of the above peptide fragments was chosen as a model for characterization of antioxidative properties with chemical synthesis of 28 structurally related peptides to investigate the structure-activity relationship (Chen *et al.*, 1997). His and Pro in the sequence were found to play important roles in the antioxidative activity; among the peptides tested. Pro-His-His was most antioxidative. The antioxidative peptides also exhibited synergistic effects with nonpeptidic antioxidants, such as BHA, BHT and α-tocopherol, as observed in the peptides of the soybean protein digest. Pepsin-solubilized elastin peptides were reported recently to show antioxidative activity as demonstrated by their ability to inhibit oxidation of oleic acid and consequently lower peroxide and carbonyl values (Hattori *et al.*, 1998). Proteins are commonly regarded as being effective antioxidants in emulsions. For example, proteins derived from meat, fish, milk and cereal grains are all capable of retarding lipid oxidation in oil-in-water emulsions (Lin *et al.*, 1993).

2.5.2 Antioxidants and disease

The food antioxidants, especially those from natural sources, are being touted as free radical scavengers that may act to prevent cancer, heart disease and cataracts (Madhavi *et al.*, 1996). Some researchers have suggested that synthetic antioxidants in the diet may also prevent cancer. Free radicals produced in the body as natural

products of oxidative reactions, can cause oxidation of cell lipids and protein as well as DNA damage that may lead to serious diseases. Dietary antioxidants are thought to scavenge these free radicals, prevent them from damaging cells and DNA and possibly reduce oxidized fatty acids or mutagens that lead to heart disease or cancer (Reische *et al.*, 1998). Epidemiological studies have shown trends suggesting that antioxidants may be beneficial in disease prevention, however, the interference of lifestyle factors makes the interpretation of such results inconclusive.

2.6 Food Flavour

Food flavours may be divided into two groups, based on the way in which they are formed. Primary flavours are considered to consist of those organoleptically active compounds present in raw foodstuffs that are synthesized by defined biochemical pathways. Secondary flavours are formed by chemical breakdown of, or interaction between various components of foods, e.g. fats, amino acids, sugars, etc., which takes place during cooking and/or maturation processes (Whitehead, 1998). Virtually all cellular constituents can be metabolized and/or degraded to one or more types of flavour compounds. Among the primary flavours, some, such as terpenes, are synthesized by purely anabolic pathways, while others, such as aldehydes and lactones, result from the catabolic breakdown of cellular constituents that occurs during normal cellular metabolism or after tissue injury. However, secondary flavours are formed by nonenzymatic processes from one or more starting materials. In some cases, the

same compounds can be formed as either a primary or secondary flavour. The Maillard reaction offers a typical example of the type of reactions which generate secondary flavour compounds that fall into one of two distinct classes of flavour-active compounds based on the source of the flavour. Natural and synthetic flavours or flavour compounds are two broad classifications based on the source of the particular flavour compound. However, the broad definition is the one given by the United States Code of Federal Regulation (1983). This defines natural flavours and natural flavourings as "the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate or any product of roasting, heating or enzymolysis which contains the flavouring constituents derived from a spice, fruit juice, vegetable or vegetable juice, edible yeast, herb, bud, bark, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products or fermentation products thereof whose significant function in food is flavouring rather than nutrition". Artificial flavours or flavourings, on the other hand, are defined as "any substance, the function of which is to impart flavour, which is not derived from the above-mentioned materials". This definition is rather broad, and leaves avenues for interpretation. However, it is clear that flavour compounds produced by enzymatic, and in some cases thermal means such as Maillard reaction, may be considered as natural.

2.6.1 Thermal flavour generation

One of the major reactions involved in the formation of food flavours during thermal processing of foods is the Maillard reaction or non-enzymatic browning

reaction which was first reported by Maillard in 1912. Most foods are made up of a complex mixture of compounds, including carbohydrates, amino acids and proteins. When foods are heated, formation of a large variety of volatile compounds takes place. The process involves reaction between the carbonyl group of reducing sugars and free amino groups of amino acids, peptides or proteins and occurs in food under appropriate reaction conditions such as heat, alkaline pH and low moisture content. In most foods, the ε-amino groups of lysine residues of proteins or peptides are the most important sources of free amino groups, and the ease with which they take part in the reaction explains why the Maillard reaction is one of the routes to nutritional damage of food proteins (Hurrell, 1989). The Maillard reaction comprises a complex network of intertwining reactions and takes place during food processing, especially when heat treatment is involved, and also during storage (Ames, 1992). Other thermal reactions such as pyrolytic degradation of the main components of foods, including sugars, amino acids, vitamins as well as the oxidative degradation of lipids also contribute to the formation of heterocyclic compounds responsible for the flavour of foodstuffs (Erickson, 1992). Phenolic substances, flavonoids and carotenoids are likely substrates for non-enzymatic browning reactions and may serve as precursors of some aromas. Heterocyclic compounds possessing some flavour notes may also be formed enzymatically, in certain foods such as vegetables (tomatoes, asparagus) and fruits (pineapple) and during the ripening of cheese and fermentation of alcoholic beverages (Vernin and Parkanyi, 1982). The reaction is responsible for both

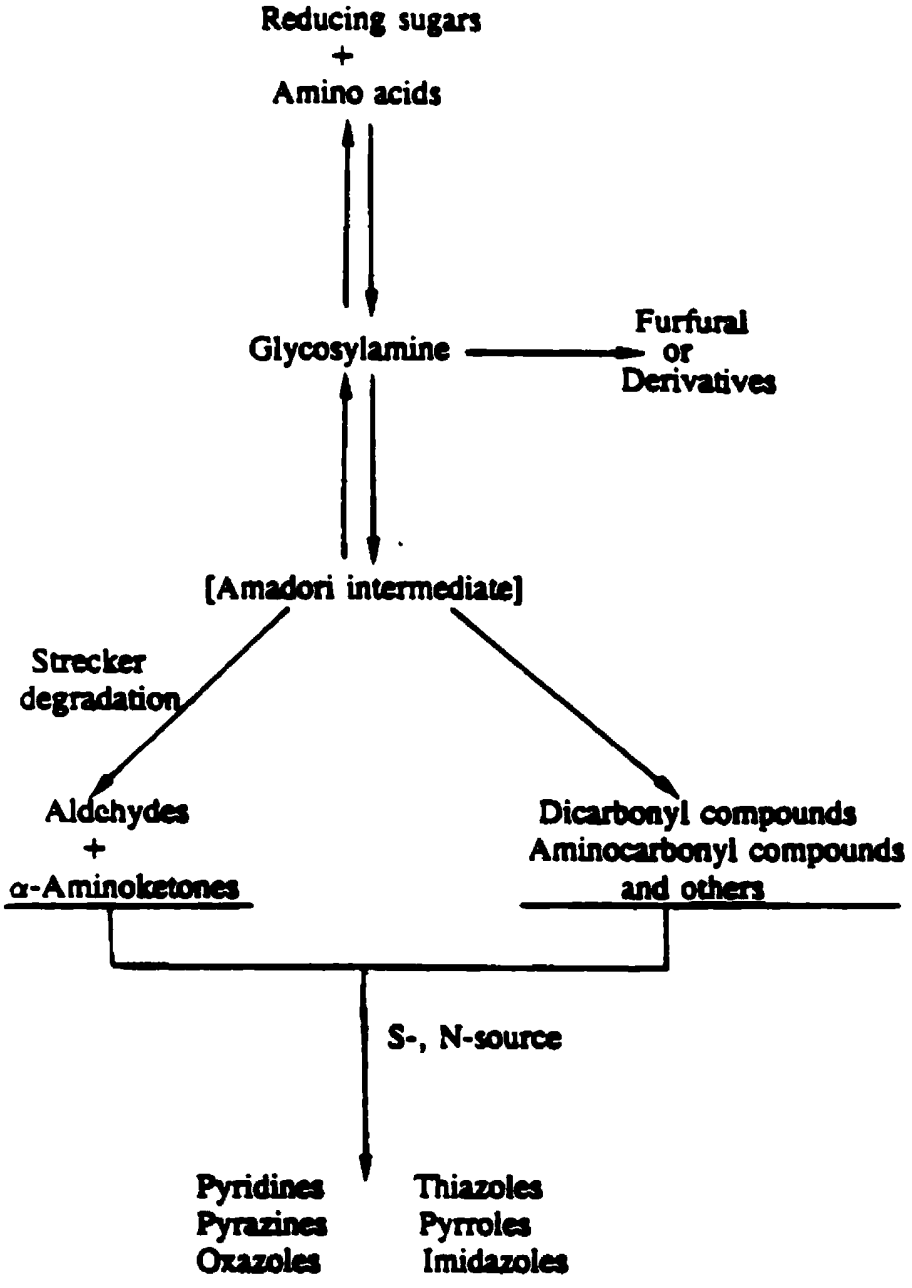
desirable and undesirable aromas and colours in foods. The aromas of bread, chocolate, coffee and meat are all examples of desirable aspects of this reaction. The aromas of burned food, canned products, stale milk powder and dehydrated potatoes are typical examples of the undesirable aspects of this reaction. The reaction has been extensively reviewed in many books and journal articles (Erickson, 1981; Waller *et al.*, 1983; Fujimaki *et al.*, 1986; Finot *et al.*, 1990; Parliment *et al.*, 1994; Ho, 1996).

The nature of the transformation of the initial stages of reactions involves the formation of a Schiff base (aldamine) between the amino and carbonyl groups. This is followed by the rearrangement of the Schiff's base to an Amadori compound (1-amino-1-deoxy-2-ketose). The Amadori compounds then react further via several pathways, including enolization, dehydration, aldol condensation and Strecker degradation, to form a large number of compounds. Chan and Reineccius (1994) reported that nearly all foods examined by aromagram methodology contained aromas characteristics of Strecker aldehydes (caramel, potato and floral note). The Strecker reaction involves oxidative deamination and decarboxylation of an α-amino acid in the presence of a dicarbonyl compound. The products formed from this reaction are an aldehyde, containing one less carbon than its original amino acid, and an α-aminoketone. The interaction of reaction products with those of the Strecker degradation leads to the formation of many additional flavour compounds. These other compounds are mainly heterocyclics and responsible for the characteristic

flavour of some heat-processed foods, such as roast meat, baked foods and toasted bakery products.

The five and six member ring heterocyclics are among the most potent flavour compounds arising from cooking. The oxygen-containing rings are most characteristic of plant foods, especially grain products, or of the lipid derived-flavour of certain foods. Popcorn and potato chips are flavoured in part by these oxygen-containing heterocyclic compounds (Ames, 1992). The complex flavours of coffee and chocolate are also in part derived from sulphur- and nitrogen-heterocyclics, as are meat flavours. Heat-induced heterocyclic sulphur and nitrogen compounds include pyridines, pyrazines, thiazoles, pyrroles and thiophenes. Relatively little is known about the nutritional properties of a large number of these heat-induced compounds. Many of these compounds are naturally occurring, although usually found at much lower levels. Others such as oxazoles, and oxazolines are unlikely to be encountered in a natural nutritional food system (Maga *et al.*, 1983). A simplified illustration of the main steps of complex reactions which lead to the formation of heterocyclic volatile flavour compounds in food is given in Figure 2.6. Flavour components formed by the Maillard reaction can best be divided into three groups: (i) Simple sugar dehydration/fragmentation products, furans, pyrones, cyclopentenes, carbonyls, acids; (ii) amino acid degradation products, aldehydes, sulphur compounds; and (iii) volatiles produced by further interactions, including pyrroles, pyridines, imidazoles, pyrazines, oxazoles, thiazoles and compounds formed from condensation reactions (Ames, 1992).

Figure 2.6 A simplified scheme for Maillard reaction.


Reducing sugars
+
Amino acids
Glycosylamine
Furfural
or
Derivatives
[Amadori intermediate]
Strecker
degradation
Aldehydes
+
α-Aminoketones
Dicarbonyl compounds
Aminocarbonyl compounds
and others
S-, N-source
Pyridines
Pyrazines
Oxazoles
Thiazoles
Pyrroles
Imidazoles

2.7 Optimization of experimental conditions

The importance of development of optimal-design theory in the field of experimental designs emerged following World War II. It was motivated by various authors (Elfving, 1952; Kiefer, 1958; Kiefer and Wolfowitz, 1959). Optimal-design theory has clearly become an important component in the general development of experimental design for the case of regression models; their application in response surface methodology (RSM) in the past three decades has been enormous. Response surface methodology, originally described by Box and Wilson (1951), is effective for responses that are affected by many factors and their interactions. RSM is essentially a particular set of mathematical and statistical methods used by researchers to aid in the solution of certain types of problems which are pertinent to scientific and engineering processes. Its greatest application has been in industrial research, particularly in situations where a large number of variables in a system influence some features of the system. This feature is termed the response, which is a variable and usually measured on a continuous scale. Also contained in the system are input variables or independent variables, which have an effect on the response and are subject to the control of the experimenter (Myers, 1971). Response surface experiments attempt to identify one or more response variables of a system as a function of the independent variables. Generally, the results of response surface experiments are reported as a mathematical model and are used to optimize the system response. The response can be thought of as a surface over the explanatory variables'

experimental space (Thompson, 1982). Response surface experiments are run for many purposes in addition to finding optimum or improved response levels. The experimental strategy and analysis in RSM revolves around the assumption that a response n is a function of a set of design variables x^1, x^2, ..., x^k and that the function can be approximated in some region of the x's by a polynomial model (Myers *et al.*, 1989). The food industry has been a prime user of RSM since the early 1970s. This is reflected in the large number of publications in food science-related journals and its industrial usage as well.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Shark (*Isurus oxyrinchus*) was bought at a local supermarket. Capelin (*Mallotus villosus*) and lumpfish (*Cyclopterus lumpus*) as well as whole Northern shrimp (*Pandalus borealis*), and the processing discards thereof, were obtained from local seafood processing plants. Whole capelin was subjected to mechanical deboning using a Baader 694 deboning machine (Baader, Lübeck, Germany) with a drum orifice size of 3 mm. Shark meat was manually deboned, after trimmings and removal of visible connective tissues, then ground using an Omega Meat Grinder (Larry Sommers Ltd. Toronto, ON). Cut up whole lumpfish without the viscera and shrimp heads were ground using an Omega Meat Grinder and homogenized immediately before use or stored at -20 °C until used (i.e. within 3 weeks).

All chemicals used were of analytical grade and were obtained from Fisher Scientific Co., Montreal, PQ, unless where otherwise specified.

3.2 pH Measurement

The pH of samples, where applicable, was measured using a Fisher Accumet pH meter model 805MP (Fisher Scientific Co., Montreal, PQ) equipped with a combination electrode.

3.3 Proximate composition

3.3.1 Moisture content

Approximately 3-4 g of original sample or 2 g of protein hydrolysate were accurately weighed into a preweighed aluminium dish and placed in a forced-air convection oven (Fisher Isotemp 300, Fair Lawn, NJ) which was preheated to 105±1 °C. Samples were held at this temperature overnight or until a constant weight was attained. The moisture content was calculated as percent ratio of the weight difference of samples before and after drying to that of the original material (AOAC, 1995).

3.3.2 Crude protein

Approximately 0.05-0.06 g of protein hydrolysate or 0.3-0.4 g of its starting material was accurately weighed on a nitrogen-free paper and placed in a digestion tube of a Büchi 430 digestor (Büchi Laboatoriums, Fawil, Switzerland). In case of shrimp heads with the shells, a known amount of their base-solubilized matter was used. The nitrogen content in different samples was determined by digestion in 20 mL of concentrated H_2SO_4 (Fisher Scientific Co.) in the presence of two Kjeltab catalyst tablets obtained from Profamo (Dorval, PQ) in the digestor until a clear solution was obtained. Digested samples in digestion tubes were diluted with 50 mL of distilled water followed by addition of 150 mL of a 25% (w/v) NaOH solution. Nitrogen in the sample was converted to ammonia which was steam-distilled using a Büchi 321 distillation unit (Büchi Laboratoriums) into a 4% (w/v) H_3BO_3 solution (50

mL) containing a few drops of end point indicator (EM Science, Gibbstown, NJ). The content of ammonia in the distillate was determined by titrating it against a 0.1M standardized H_2SO_4 solution (AOAC, 1995). The crude protein content in the sample was calculated as %N x 6.25.

3.3.3 Determination of total lipids

Total lipids were extracted into a mixture of chloroform and methanol as described by Bligh and Dyer (1959). Approximately 25 g of sample were accurately weighed and extracted with a mixture of 25 mL chloroform and 50 mL methanol (1:2 v/v) by homogenizing for 3 min using a Polytron homogenizer Model PT-3000 (Brinkmann Instruments, Rexdale, ON) at speed level of 4. A further extraction was carried out with the addition of 25 mL chloroform followed by homogenization using a Polyton homogenizer. About 25 mL of distilled water were added and the mixture was then suction-filtered through a Büchner funnel using a Whatman filter paper No 3 (Fisher Scientific Co.). Dilution with chloroform and water resulted in separation of homogenate layers and inclusion of lipids in the chloroform. The filtrate was separated overnight in a separatory funnel. A 10 mL aliquot of the lipid extract in chloroform, after drying over anhydrous sodium sulphate, was transferred into a tared 50 mL round bottom flask and the solvent was removed at 35 °C under vacuum using a Büchi RE 111 rotavapor (Büchi Laboratoriums, Fawil, Switzerland). The flask was then placed in a forced-air convection oven at 80 °C for 1 h. After cooling in a desiccator, the lipid content was determined gravimetrically.

3.3.4 Ash content

Approximately 3-4 g of sample were weighed into a cleaned porcelain crucible and then charred over a Bunsen burner and subsequently placed in a temperature controlled Thermolyne 62700 muffle furnace (Barnstead/Thermolyne, Dubuque, IA) which was preheated to 550 °C. Samples were held at this temperature until a grey ash was produced and then allowed to cool in a desiccator and weighed immediately. Ash content was calculated as percent ratio of the weight of the ash obtained after ignition to that of the original material (AOAC, 1995).

3.4 Preparation of thermally stable protein dispersions

3.4.1 Sample preparation

Capelin and shark samples were prepared as reported in section 3.1. Mechanically deboned capelin meat or ground shark (500 g) was suspended in 1000 mL cold water and stirred gently over a 30 min period. The suspension was then filtered through three layers of cheesecloth to remove the wash water. The residue was then suspended in 1000 mL of a 0.5 % (w/v) solution of sodium chloride and then in a 0.5% sodium bicarbonate solution. After a 30 min standing, the residue was collected as before and the resultant meat was further washed with water to remove any residual bicarbonate. The resultant washed meats were used for further studies.

The washed meat was suspended in cold water at proportions ranging from 10 to 50% (w/v) and then homogenized in a Waring blendor (Model 33BL73; Dynamic

Corp., New Hartford, CT). The homogenate was held in a water bath at 70 or 100 °C for 15 min and subsequently cooled to room temperature and centrifuged at 2675 x g for 20 min using an IEC CENTRA MP4 centrifuge (International Equipment Co., Needham Heights, MA). The supernatant was collected and its protein content determined as described in Section 3.3.2. A flowsheet summarizing steps involved in production of protein dispersions is given in Figure 3.1.

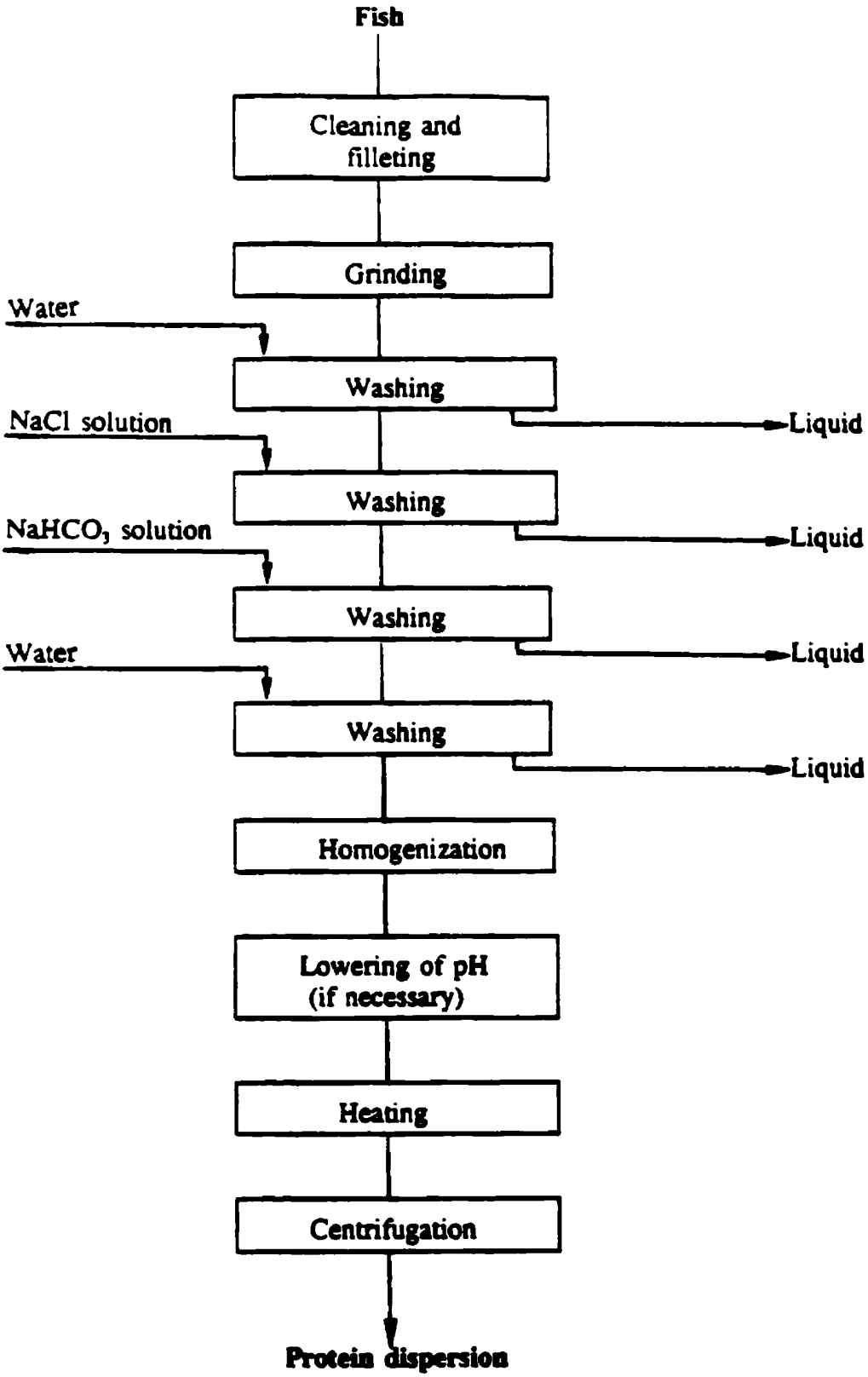
3.4.2 Thermal stability

Aliquots (20 mL) of the dispersion were transferred to screw-capped glass vials. The vials were then held in a boiling water bath for 15 min. After cooling to room temperature, the vials were centrifuged at 2675 x g for 15 min. The content of protein in the supernatant was then determined as given in section 3.2.2.

3.4.3 Stability to pH variation and salts

The pH of the dispersion was adjusted between 3.5 and 10 by addition of a 2M solution of HCl or NaOH. The samples were then heated in a boiling water bath for 15 min, cooled to room temperature and centrifuged at 2675 x g for 15 min. The content of protein in the supernatant was then determined as described in section 3.3.2. In order to examine the effect of salts on thermal stability of the protein dispersion, known volumes of 1M NaCl, KCl or $CaCl_2$ were added to the dispersion in order to obtain a 0 to 50 mM salt solution. After thorough mixing, the samples

Figure 3.1 Flowsheet for the preparation of thermostable protein dispersions.


Fish
Cleaning and filleting
Grinding
Water
Washing
NaCl solution
Liquid
Washing
NaHCO3 solution
Liquid
Washing
Water
Liquid
Washing
Liquid
Homogenization
Lowering of pH (if necessary)
Heating
Centrifugation
Protein dispersion

were heat-treated in a boiling water bath for 15 min. cooled and centrifuged at 2675 x g for 20 min. The percentage of protein remaining in the supernatant was then determined. In another set of experiments. the influence of sodium tripolyphosphate (STPP) on the solubility characteristics of dispersed proteins was tested. The concentration of STPP in the dispersion was 25 mM. After mixing, the samples were heat-processed, cooled and centrifuged as described above.

3.4.4 Viscosity measurements

The viscosity of dispersions was measured using a Brookfield syncro-lectric viscometer. model LVT (D.W. Brookfield Ltd., Cooksville, ON), calibrated with a Brookfield standard having a viscosity of 98.2 cps. During measurements. the dispersion (200 mL) was held in a water bath at the desired temperature. The viscosity was measured routinely at 60 rpm using spindle No. 2 or 3. The values were recorded after 1 min of rotation of spindle in the dispersion. Viscosity values were obtained from the conversion factor provided by the manufacturer (Tung, 1978). The apparent viscosity (Rao, 1977) values were expressed in Pascal seconds (Pa.s). In case of shark protein dispersions, viscosity was measured before acidification and immediately after the addition of glacial acetic acid (1 mL) to 200 mL dispersion as well as after heating at 100 °C over a period of 0 to 30 min followed by cooling of the dispersions (200 mL) to room temperature.

3.4.5 Effect of heating time on solubility of protein dispersion

Both shark and capelin were used for preparation of protein dispersions, with protein contents of 1.14 and 2.27 % (w/v), respectively. Acidified shark dispersions and unacidified capelin dispersions were heated for 10, 20, 25, and 30 min at 100 °C, and the protein remaining in solution after heat treatment, expressed as percentage of the total protein in the unheated dispersion, was determined following cooling and centrifugation.

3.4.6 Effect of protein concentration and temperature on apparent viscosity

The effect of protein content of the unacidified and acidified protein dispersions was examined by determining the viscosity at varying temperatures of 5, 15, 25, 30, 35 and 45 °C for shark dispersions. For studying the effect of protein concentration of the unacidified and acidified dispersions, varying amounts of washed meats were dispersed in water to obtain protein concentrations of 1.05, 1.30, 1.83 and 2.27% in case of capelin and 0.38, 0.76, 1.14 and 1.52% in case of shark protein dispersions.

3.5 Tristimulus colour parameters

The tristimulus colour parameters, namely Hunter **L** (100, white; 0, black), **a** (+, red; -, green) and **b** (+, yellow; -, blue) values of the samples were determined by surface reflectance measurements using a Colormet colorimeter (Instrumar Engineering

Limited, St. John's, NF). The unit was standardized with a B-143 white calibration tile with Hunter values **L**, 94.5 ± 0.2; **a**, -1.0 ± 0.1; and **b**, 0.0 ± 0.2. Measurements were made on a 20 mm thick layer of sample, in a sealed 16.5 cm x 16 cm transparent polythylene pouches (Eastern Paper Co., St. John's, NF). Generally 6 readings were recorded at different areas of the sample surface.

3.6 Preparation of protein hydrolysates

The ground shrimp heads or lumpfish samples were mixed with water (at appropriate ratios) and homogenized in a Waring blendor (Model 33BL73; Dynamic Corp.) for about 2 min at room temperature. The pH was adjusted to optimum activity value for each enzyme. The hydrolysis was performed under different conditions with respect to temperature, pH, meat to water ratio and concentrations of substrate and enzyme in a 600 mL reaction vessel. The pH of the mixture was kept constant by continuous addition of a 4M NaOH solution to the reaction mixture. The reaction was stopped by heating the mixture at 90 °C for 15 min in order to inactive the enzyme. The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds broken to the total number of bonds per unit weight, was calculated from the amount of base consumed (Adler-Nissen, 1986a) as given below:

$$DH\ \% = [(B * M_b)/(\alpha * M_p * h_{tot})] * 100$$

where B value is the amount of base consumed in litre; M_b, molarity of base used in

pH stat hydrolysis process; M_p, mass of protein (N x 6.25) in kg; h_{tot} was estimated by adding up the amount of individual amino acids (mmoles) per gram of protein of raw material; α, average degree of dissociation of the α-NH_2 groups released during hydrolysis and expressed as:

$$\alpha = (10^{pH-pK})/(1 + 10^{pH-pK})$$

where pK values at different hydrolysis temperatures. T, were calculated according to Steinhardt and Beychok (1964) using the equation:

$$pK = 7.8 + [(298 - T)/(298 * T)] * 2400$$

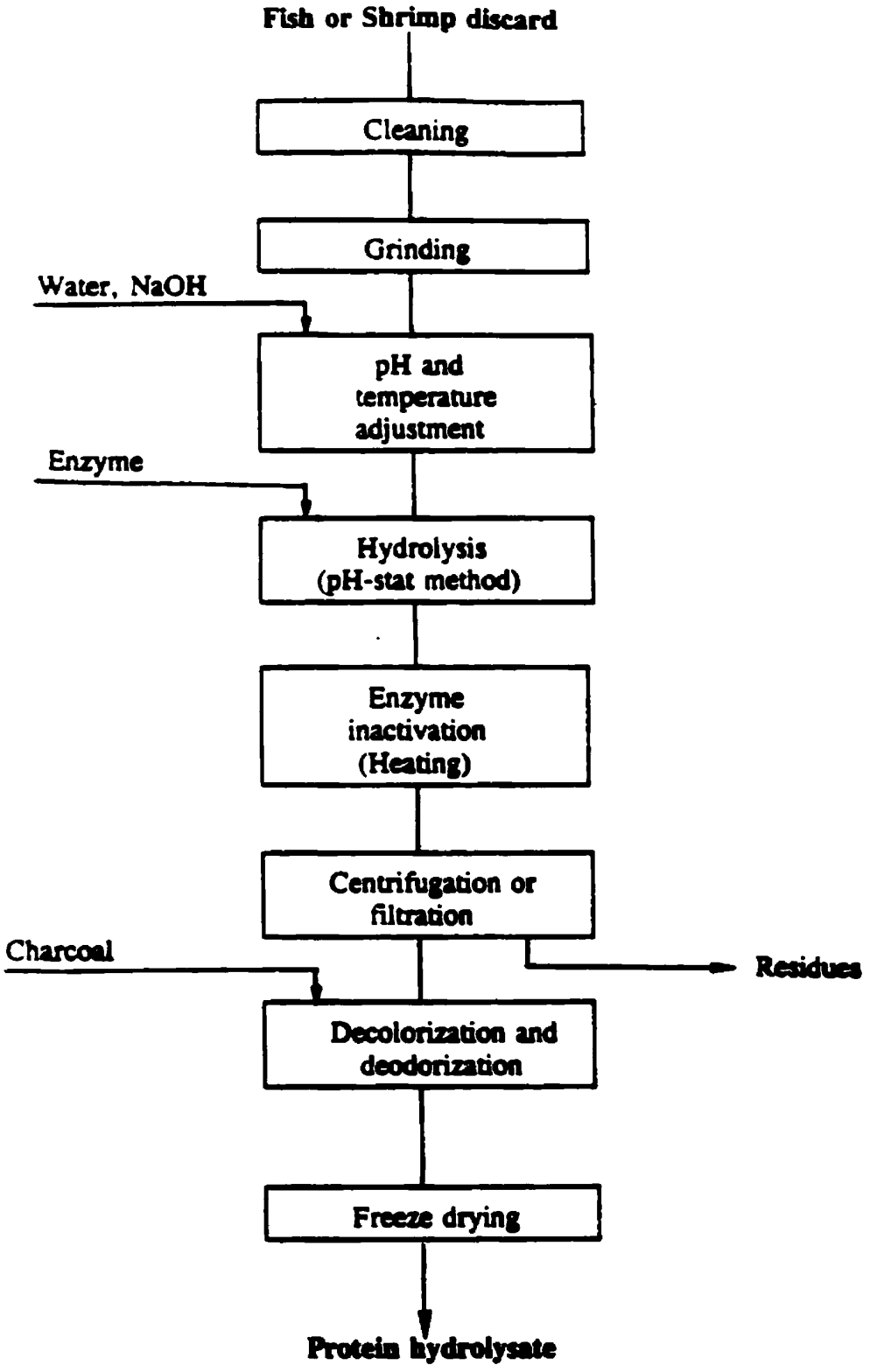
where T is the hydrolysis temperature in K.

The sludge, after the hydrolysis process, was removed by suction filtration. Removal of coloured and odouriferous matter was achieved using fine grain charcoal (Aldrich, Milwaukee, WI) at 1 - 2% (w/v) and stirring at 55 °C for 30 min. The decolourized hydrolysate was dehydrated at -45 °C for 24 h using a Labconco Freezone freeze dryer (Labconco Corporation, Kansas, MO). The ratio of total analyzable Kjeldahl nitrogen in the final product to that originally present in the starting material was calculated as the percent nitrogen recovered. A flowsheet of the main steps involved in the preparation of protein hydrolysate is given in Figure 3.2.

3.6.1 Enzyme studies

The enzymes used in this study were trypsin (Sigma Chemical Co., St. Louis, MO), as well as Neutrase 0.5L and Alcalase 2.4L (Novo Industrie AS, Bagsvaerd,

Figure 3.2 Flowsheet for the preparation of protein hydrolysates by enzymatic hydrolysis.


Fish or Shrimp discard
Cleaning
Grinding
Water, NaOH
pH and temperature adjustment
Enzyme
Hydrolysis (pH-stat method)
Enzyme inactivation (Heating)
Centrifugation or filtration
Residues
Charcoal
Decolorization and deodorization
Freeze drying
Protein hydrolysate

Denmark). Preliminary experiments were carried out to determine the most suitable enzyme amongst those considered for hydrolysis of shrimp proteins. The optimum application conditions of the enzymes in literature (see Table 3.1), or those supplied by the manufacturers, were used in the preliminary experiments.

3.6.2 Enzymatic hydrolysis of ground shrimp heads and lumpfish for selection of protease

Ground shrimp heads were mixed with water at a ratio of 1:2 (w/v) in a 400 mL beaker and preincubated for 20 min at desired temperature (40 - 65 °C) in a thermostated water bath. The pH of the medium was adjusted with 2M NaOH or HCl followed by the addition of the appropriate enzyme (trypsin, Neutrase or Alcalase) to the reaction vessel and mixing vigorously using a mechanical stirrer (Caframo, Wiarton, ON) at 250 rpm. The hydrolysis was carried out under constant pH; the three enzymes used and their characteristics are given in Table 3.1.

3.7 Optimization experiments for the production of protein hydrolysates

3.7.1 Experimental design and data processing

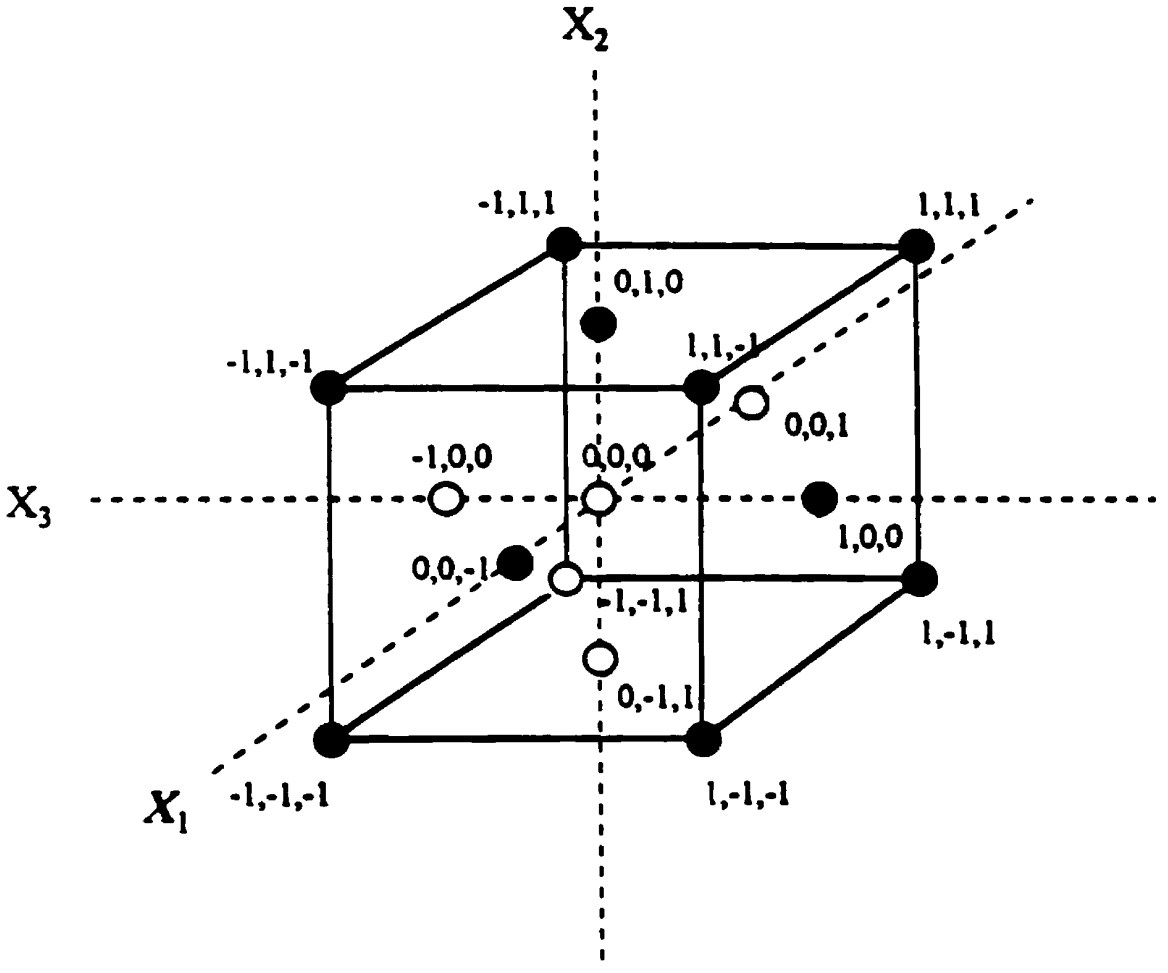
Response surface methodology (RSM) was used to optimize hydrolysis parameters such as pH, temperature and enzyme/substrate concentration ratio. Optimization experiments were carried by employing a three-factor face centred cube design (FCCD) of Box and Behnken (1960) given in Figure 3.3. Based on preliminary experiments, independent variables, namely temperature (X_1), pH (X_2) and

Table 3.1 Characteristics of proteases used in this study[a].

Common name	Source	Type of protease	Typical pH range	Preferential specificity[b]
Neutrase	*Bacillus amyloliquefaciens*	Metalloprotease	6-8	Leu-Phe-NH_2, and others
Alcalase	*Bacillus licheniformis*	Serine protease	6-10	Broad specificity, mainly hydrophobic -COOH
Trypsin	Ox, pig	Serine protease	7-9	Lys-Arg-COOH

[a] Adler-Nissen (1986a).
[b] Terminal amino acid after cleavage.

Figure 3.3 Face-centred cube design for three variables (points are given as coded variable forms).


X2
X3
X1
-1,1,1
1,1,1
0,1,0
-1,1,-1
1,1,-1
0,0,1
-1,0,0
0,0,0
1,0,0
0,0,-1
-1,-1,1
1,-1,1
0,-1,1
-1,-1,-1
1,-1,-1

enzyme concentration (X_3) were studied to optimize the degree of hydrolysis (Y_1) and nitrogen recovery (Y_2) as responses (Y variables). Enzyme/substrate concentration ratio was expressed in Anson units per kg crude protein. One Anson unit (AU) is the amount of enzyme which under the given reaction conditions digests haemoglobin at an initial rate such that there is liberated per 10 min an amount of trichloroacetic acid-soluble reagent as 1 μmol of tyrosine (Adler-Nissen, 1986a). The independent variable values were chosen based on preliminary experiments. For each factor, three levels were selected and the range of these variables are shown in Table 3.2. The coded values were obtained using the formula:

$$E = (D - D_1)/\Delta D$$

where E is the coded value, D is the corresponding uncoded value, D_1 is the uncoded value in the centre of the domain, and ΔD is the increment of D corresponding to one unit of E. Table 3.3 shows the experimental domains involving independent variables and their levels in coded and uncoded forms used in the enzymatic hydrolysis experiments. These consisted of 18 experiments for the study of three experimental design factors. Duplicate experiments were carried out at all design points, except at the centre point (0,0,0) where four replicates were performed in order to estimate the residual variance. A second order (quadratic) polynomial model was assumed for predicting Y variables and given as:

$$Y = \beta_o + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\beta_{ii} x_i^2 + \sum_{i<j=1}^{3}\sum\beta_{ij} x_i x_j$$

where β_o, β_i, β_{ii} and β_{ij} are intercept, linear and quadratic and interaction regression

Table 3.2 Independent variables and experimental design levels expressed in coded and uncoded forms for shrimp heads and lumpfish protein hydrolysis.

Material	Code unit	Independent variables		
		Temp (°C)	pH	E/S[1]
Shrimp heads	-1	45	7	20
	0	55	8	30
	+1	65	9	40
Lumpfish	-1	45	7	15
	0	55	8	25
	+1	65	9	35

[1]Enzyme/substrate concentration ratio (AU/kg crude protein).

Table 3.3 Face-centred cube design (FCCD) consisting of 18 experiments for the study of three experimental factors in coded units.

Run no.	Temp. (°C)	pH	E/S[a]
1	-1	-1	-1
2	-1	-1	1
3	-1	1	-1
4	-1	1	1
5	1	-1	-1
6	1	-1	1
7	1	1	-1
8	1	1	1
9	-1	0	0
10	1	0	0
11	0	-1	0
12	0	1	0
13	0	0	-1
14	0	0	1
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0

[a] Enzyme/substrate concentration ratio (AU/kg crude protein).

coefficient terms, respectively, and x_i and x_j are independent variables. The Statistical Analysis System (SAS, 1990) was used for multiple regression analysis, analysis of variance (ANOVA), canonical analysis and analysis of ridge maximum of data in the response surface regression (RSREG) procedure. The full model fitted polynomial regression equations, obtained from RSREG analysis, were expressed graphically in three dimensional and contour plots to visualize the relationships between the responses and independent variables. These were performed by holding the independent variables with the least effect on the response at a constant value and changing the levels of the other two variables.

3.8 Total amino acid composition

The amino acid composition of lyophilized and powdered samples was determined by digestion in 6M HCl at 110 °C for 24 h (Blackburn, 1978). The amino acid composition of the resultant hydrolysate was determined using a Beckman 121 MB amino acid analyzer (Beckman Instruments Inc., Palo Alto, CA). Tryptophan was determined after 24 h hydrolysis of the protein at 100 °C in the presence of 3M mercaptoethane sulphonic acid (Penke *et al.*, 1974). For determination of methionine and cysteine, samples were subjected to performic acid oxidation prior to hydrolysis in 6M HCl (Blackburn, 1978).

3.8.1 Calculation of protein efficiency ratio, amino acid score and biological value

Protein efficiency ratio (PER) values of shrimp heads and protein hydrolysates were calculated according to the equations developed by Lee *et al.* (1978), as given below:

PER calculated = 0.06320 [ΣAA_7] - 0.1094 Equation 1

PER calculated = 0.06320 [ΣAA_{10}] - 0.1539 Equation 2

where ΣAA_7 = THR + VAL + MET + ILE + LEU + PHE + LYS

$\Sigma AA_{10} = \Sigma AA_7$ + HIS + ARG + TRP

The amino acid score (or chemical score) is the content of each essential amino acid in a food protein expressed as a percentage of the content of the same amino acid in the same quantity of a protein selected as a standard (FAO/WHO, 1991). The standard used was amino acid scoring reference recommended by FAO/WHO/UNU (1985).

Predicted biological value (BV) was calculated according to the equation derived from regression analysis reported by Mørup and Olselen (1976) and given below.

$$BV = 10^{2.25} \times q^{0.41}_{Lys} \times q^{0.60}_{Phe+Tyr} \times q^{0.77}_{Met+Cys} \times q^{2.4}_{Thr} \times q^{0.21}_{Trp}$$

Where, $q = a_{sample}/a_{reference}$ for $a_{sample} \leq a_{reference}$

or $q = a_{reference}/a_{sample}$ for $a_{sample} \geq a_{reference}$

a = mg of amino acid per g of total essential amino acids.

3.9 Free amino acid composition

The content of free amino acids in the samples was determined by homogenizing (Polytron homogenizer, speed 4) 10 g of the sample, or 1 g in case of protein hydrolysate, with 20 mL ice-cold 6% (w/v) perchloric acid. After 30 min incubation at 0 °C, samples were centrifuged at 3000 x g for 10 min at 5 °C. The procedure was repeated twice and the pH of the combined supernatants adjusted to 7.0 using a 33% (w/v) KOH solution. Perchlorate precipitates were removed after centrifugation for 10 min at 3000 x g. The supernatant was acidified with 10M HCl to 2.2, diluted at a ratio of 1:1.5 (v/v) with 1.0% (w/v) lithium citrate buffer, pH 2.2. The content of free amino acids was determined using a Beckman 121 MB amino acid analyzer (Beckman Instruments Inc.).

3.10 Functional properties of protein hydrolysates

3.10.1 Solubility

The solubility of protein hydrolysate was determined according to the method of Chobert *et al.* (1988) with minor modifications. A sample of protein hydrolysate (1%, w/v) was dispersed in distilled water and its pH adjusted by addition of a 6M HCl or NaOH solution. After a 3 min standing at room temperature, the mixture was centrifuged at 12,000 x g for 20 min. The solubility was determined by measuring the content of crude protein in the supernatant (AOAC, 1995) and expressed as percent soluble protein at a given pH.

3.10.2 Moisture and fat adsorptions

For the determination of the moisture adsorption, 1 to 2 g of accurately weighed sample was placed, as a thin layer, at 22 °C in an open aluminum tray at a given relative humidity (RH%) for a period of 24-48 h. The maximum percent weight gain was then recorded (Dev and Quensel, 1986). For fat adsorption determination, 2 g sample were transferred into a 50 ml centrifuge tube followed by addition of 12 mL corn oil and thorough mixing with a glass rod. The sample was then kept for 30 min. while mixing every 5 min for 30 s, after which it was centrifuged for 25 min at 2,000 x g. Free oil was decanted and the fat adsorption of the sample determined from the weight difference data.

3.10.3 Water holding capacity and cooking yield of meat model systems

A 20 g sample of freshly ground meat, with or without added hydrolysates or polyphosphates, were transferred into a pre-weighed, 50 mL centrifuge tube and homogenized with 20 g water. The polyphosphates used were sodium tripolyphosphate (STPP), tetrasodium pyrophosphate (TSPP) and sodium hexametaphosphate (SHMP), from Albright and Wilsons Americas (a Division of Tenneco Canada Inc., Toronto, ON). Tubes were covered with aluminum foil and placed in a boiling water bath for 20 min. The mixture was then cooled to room temperature and centrifuged for 15 min at 3,000 x g. The juice released was decanted and the sample blotted over a Whatman No. 1 filter paper and transferred back into

the tube. The percent cooking yield was calculated from the weight difference data.

In another set of experiments, to 150 g of mechanically deboned chicken meat in Mason jars, and shrimp protein hydrolysate, STPP, TSPP or SHMP were added at a concentration ranging from 0 to 3% (w/w of meat). The samples were mixed manually with a glass rod for 10 min and then stored for 1 h at 4 °C. Sealed jars were thermally processed at 80±1 °C for 40 min and cooled under a stream of cold tap water for 30 min. Seals were then removed and the drip volume measured. Crude protein concentration in the drip liquid was calculated based on the nitrogen content as determined by Kjeldahl procedure (i.e. %N x 6.25) as given in Section 3.3.2 .

3.10.4 Emulsifying properties

To a 3.5 g sample of protein hydrolysate, 50 mL distilled water and 50 mL corn oil were added. The mixture was then homogenized for 30 s using a Polytron homogenizer (setting 4) and divided into 4 equal parts in 4 centrifuge tubes. The tubes were then centrifuged at 2,000 x g for 5 min and the emulsifying capacity (EC) was calculated as the ratio of emulsified versus the total volume.

Emulsion stability of the samples was determined by heating the mixtures for 30 min at 80 °C followed by dividing them into 4 equal portions in 4 centrifuge tubes. Tubes were then centrifuged at 2,000 x g for 5 min. The volume of emulsion as well as the total volume were recorded. Emulsion stability was expressed as percent emulsifying capacity, following heating.

3.10.5 Whippability and foaming stability

Protein hydrolysate (3 g) was dispersed in 100 mL distilled water and the mixture homogenized for 1 min using a Polytron homogenizer at setting 4. The mixture was then poured into a 250 mL graduated cylinder and the total volume recorded. Whippability was expressed as percentage volume increase upon whipping; foaming stability was calculated as the volume of the foam remaining after 0.5, 20 and 30 min quiescent periods.

3.11 Gel Electrophoresis of shrimp protein hydrolysates

To the reaction mixture containing ground shrimp heads in water at a ratio of 2:1 (w/v), was added Alcalase. The reaction was carried out under optimum conditions for different hydrolysis periods (5, 10, 30, 60, 90 and 120 min). At the end of each hydrolysis time, an aliquot of the hydrolysate was taken out of the reaction vessel and transferred into a test tube and heated in a boiling water bath for 15 min to stop the reaction. The undigested material was separated from the hydrolysate by filtration through a Whatman No 3 filter paper. The filtrates were freeze-dried at -45 °C for 24 h. The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was run using an ECPS 3000/150 (Parmacia LKB Biotechnology, Baire d'Urfe, PQ) according to the method of Laemmli (1970) using a 5% stacking gel and a 10% separating gel. Five microlitres of a solution of the hydrolysate containing 1 - 2 μg of protein were applied to the gel. Proteins were stained using a 0.1%

Coomassie brilliant blue R-250 for 6 h at room temperature and destained using a mixture of 30% methanol and 10% acetic acid (1:1, v/v). Relative migration of the protein bands was estimated using LKB molecular weight markers (Pharmacia LKB Biotechnology) which were pentamer (71,500), tetramer (57,200), trimer (42,900), dimer (28,600) and monomer (14,300) of proteins with known molecular weights.

3.12 Capillary Zone Electrophoresis of shrimp protein hydrolysates

To prepare the samples for capillary zone electrophoresis (CZE), a solution of the standard protein or hydrolysate was heated at 100 °C for 15 min. Prior to heating, to a 400 μL vial were added 0.1 to 1 mg protein, 100 μL sample buffer (SDS-200 gel buffer from SDS 14-200 kit, Beckman Instruments, Palo Alto, CA), 10 μL standard Orange G dye, 5 μL 2-mercaptoethanol and 85 μL deionized water which had been filtered through a 0.2 μm filter (Micron Separations Inc., Westborough, MA). The mixture in a closed vial was stirred using a vortex until the protein dissolved. The heated mixture was cooled in an ice bath to room temperature prior to injection. The sample was injected to the capillary column over a 60 s period at 0.5 psi. The analysis was carried out using a Beckman P/ACE system 5510 (Beckman Instruments) equipped with a capillary column of 47 cm separation length and 100 μm internal diameter, thermostated at 20 °C. SDS sample buffer was used at 14.1 kV and average current of 40 μA. Detection was achieved with a diode array detector which allowed the recording of UV spectra at 214 nm to produce the electrophoretograms. The

electrophoretic comparisons of the migration times (relative mobilities) of peptides with those of authentic markers were performed to estimate the molecular weights of different peptide fractions. Electrophoresis calibration kit for molecular weight determination of polypeptides (Pharmacia Biotech Inc., Baie d'Urfe, PQ) was used as markers. These were myoglobin and its fragments with molecular weights of 17,200, 14,600, 8,240, 6,280 and 2,560 Da.

3.13 Evaluation of antioxidant properties of protein hydrolysate in meat model systems

Antioxidant activity of a typical protein hydrolysate was evaluated in a meat model system. Ground pork was homogenized with 20% water (w/w) and 0.5 to 3.0% (w/w) protein hydrolysate. Mixtures were subsequently cooked to an internal temperature of 75 °C for 40 min in a thermostated water bath. After thermal processing, samples were cooled to room temperature and homogenized in a Waring blendor (Dynamic Inc.) followed by storage in plastic bags at 4 °C until used (at day 0, 1, 3 and 5).

The oxidative state of samples containing protein hydrolysates was evaluated by the 2-thiobarbituric acid (TBA) test according to the distillation method of Tarladgis *et al.* (1964). In all cases, a 10 g sample of freshly ground and homogenized meat was transferred into a 500 mL round bottom flask containing 97.5 mL distilled water and 2.5 mL 4M HCl solution along with a few drops of DOW

antifoam A (Fisher Scientific Co.) and several glass beads. The slurry was then distilled under atmospheric pressure and 50 mL of the distillate were collected over a 20 min period. Equal volumes (5 mL each) of each of the distillates were mixed with a 5 mL of 0.02M aqueous solution of the TBA reagent in 50 mL capped tubes and subsequently heated in a boiling water bath for a 35 min period. After cooling, the absorbance of the resultant pink-coloured chromogen was read at 532 nm.

To convert the absorbance readings at 532 nm to TBA numbers, 1,1,3,3-tetramethoxypropane, a precursor of malonaldehyde, (Fisher Scientific Co.) was used to construct a standard curve. An appropriate conversion factor was obtained from equation of the straight line (Appendix A1) and used for converting the A_{532nm} values to the so-called TBA numbers, defined as mg of malonaldehyde equivalents per kg of samples. Inhibition of TBARS formation (%) was calculated as 1-[TBA number of treated sample/TBA number of untreated sample] x 100.

3.14 Chromatographic separation and evaluation of antioxidant activity of peptide fractions of protein hydrolysates

3.14.1 Column chromatography

Approximately 3 g of prepared protein hydrolysate were dissolved in distilled water and applied to a Sephadex G-15 packed glass column (2.5 x 25 cm). Water was used as the eluting solvent. At a flow rate of 2 mL/min, the eluate was collected in 3 mL fractions in 10 mL glass tubes using a fraction collector (Pharmacia, Uppsala,

Sweden). The absorbance values of the eluate were read at 220 and 280 nm. Based on the absorbance values for the contents of different tubes, they were pooled together into major subfractions (I to V). The contents of these subfractions were freeze-dried and used for subsequent high performance liquid chromatographic analysis.

3.14.2 High performance liquid chromatography

The peptides fraction IV obtained in the column chromatography in the preceding section was further separated by semi-preparative HPLC. A Shimadzu chromatographic system (Kyoto, Japan) was used: LC-6A pump, SPD-6AV UV-VIS spectrophotometric detector, SCL-6B system controller and CR 501 chromatopac. Conditions of separation were: the column used was a Particil 10 ODS-2 column (9.4 mm x 250 mm, 10 μm, Whatman) with a precolumn (4.6 mm x 125 mm, Whatman) packed with Pellicular ODS (C_{18} groups chemically bonded to 37 - 53 μm glass beads. Whatman). A stepwise gradient solvent system of water-acetonitrile containing 5% trifluoroacetic acid (w/v) was used. At time zero, HPLC-grade water containing 5% trifluoroacetic acid (v/v) was used. The flow rate was 1.5 mL/min, and the concentration of the lyophilized hydrolysate fraction obtained via column separation was 3 mg/mL water. The column was washed with HPLC-grade water. Fraction IV obtained from Sephadex G-15 column chromatography, which exhibited most antioxidative activity in a β-carotene-linoleate model system was used for the preparative HPLC. The sample was dissolved in the final solvent, that is 40%

acetonitrile in water, and filtered through a 0.45 μm filter (Micron Separations Inc., Westborough, MA). Reverse-phase HPLC was used for preparative purposes. The concentration of this fraction was 10 mg/mL and 0.5 mL of this solubilized fraction in 40% acetonitrile was injected manually. The concentrations of acetonitrile used were 0, 10, 30 and 40%.

3.14.3 Thin layer chromatography

Two-dimensional thin layer chromatographic (TLC) separation of shrimp protein hydrolysates was carried out on silica gel high-performance TLC plates (Sigma Chemical Co.) with a propanol/water (7:3, v/v) mixture, followed by an n-butanol/acetic acid/water (4:1:2, v/v/v) system (Matsumoto *et al.*, 1976). Spots from the peptides were visualized on plates after spraying with a 0.2% ninhydrin in acetone. A second set of plates was sprayed with a β-carotene-linoleate solution (Phillip, 1974) in order to evaluate the effect of separated compounds on the oxidative state of the sprayed solutions.

3.15 Thermal reaction, isolation and analysis of flavour compounds of shrimp protein or its hydrolysates with glucose

3.15.1 Thermal reaction and isolation of compounds

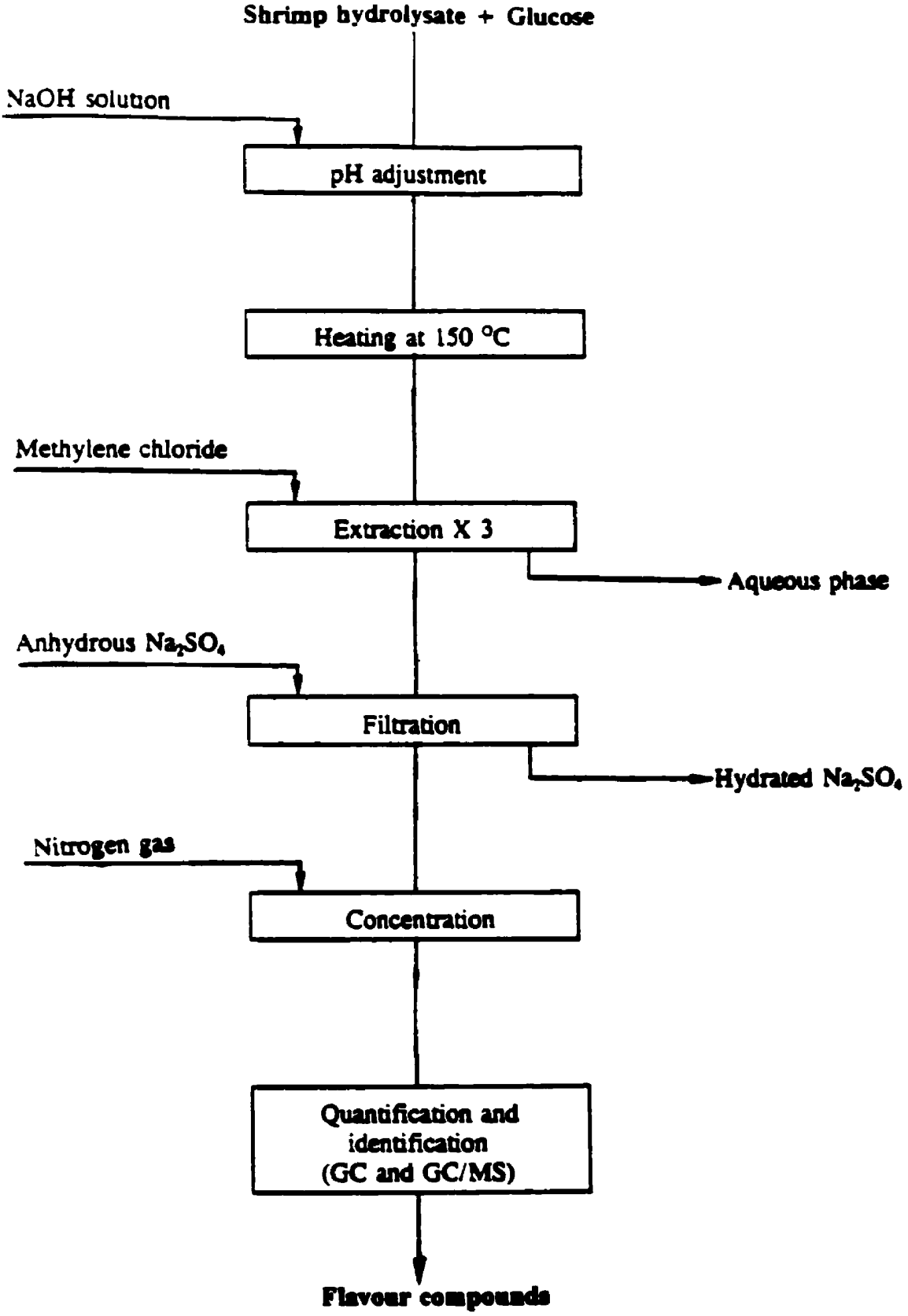
Three grams of protein hydrolysate with the addition of 0.9 g glucose were dissolved in 200 mL distilled water and put in reaction vessels. The pH was adjusted

to 7.0 by addition of 1M NaOH. The thermal reactions were carried out in a Parr reactor (Brinkmann Instruments Inc., Westbury, NY) at 150 °C for 2 h and stirred at a speed of 140 - 150 rpm. After the reaction, the pH of the mixture was adjusted back to 7.0 and 0.5 mL of 100 ppm tridecane in trichloromethane, as internal standard, were added to it. The reaction mixture was then extracted three times with 40 mL methylene chloride. The organic phase was dried over anhydrous sodium sulphate before being concentrated to about 1-2 mL with a gentle stream of nitrogen. The concentrated extract was then used for gas chromatographic (GC) and gas chromatography-mass spectrometric (GC/MS) analysis. A flowsheet for the preparation, isolation, quantification and identification of thermally-generated flavour compounds is given in Figure 3.4.

3.15.2 Gas Chromatographic (GC) analysis

A Varian 3400 GC equipped with DB-WAX column (I.D. 0.25 mm, 30 m. 0.25 μm film thickness, J & W Scientific) and a flame ionization detector (FID) was used to separate and quantify the volatile compounds. The injector and detector temperatures were set at 200 and 230 °C, respectively. The oven temperature was programmed linearly from 40 to 210 °C at 2 °C/min, and then held at 210 °C for 30 min. A split ratio of 25:1 was used. The flow rate of the carrier gas, helium, was 1 mL/min. Linear retention indices of the volatile components were calculated with n-paraffins (C6 - C25) as references (Majlat *et al.*, 1974). The paraffin standards were analyzed using the same GC conditions (section 3.20.2).

Figure 3.4 Flowsheet for the preparation, isolation, quantification and identification of thermal flavour compounds of reaction involving shrimp protein or its hydrolysates and glucose.


Shrimp hydrolysate + Glucose
NaOH solution
pH adjustment
Heating at 150 °C
Methylene chloride
Extraction X 3
Aqueous phase
Anhydrous Na_2SO_4
Filtration
Hydrated Na_2SO_4
Nitrogen gas
Concentration
Quantification and identification (GC and GC/MS)
Flavour compounds

3.15.3 Gas chromatography-mass spectrometric (GC-MS) analysis

The GC-MS analysis was performed on a Varian 3400 gas chromatograph coupled with a Finnigan Mat Ion Trap Detector (Finnigan Mat. San Jose. CA). A DB-WAX column (I.D. 0.25 mm, 30 m, 0.25 μm film thickness, J & W Scientific) was used. The operation conditions were the same as those used in GC analysis.

3.15.4 Identification and quantification of volatile compounds

Identification of volatile compounds in the condensate was based primarily on GC-MS analysis, and comparison of the GC retention indices with those of C6-C25 n-paraffins as references. The compounds were quantified using chromatogram of GC/FID. The concentration of compounds was calculated on a weight basis. The structure assignment of volatile compounds was accomplished by comparing the mass spectral data of individual compounds with those of authentic compounds available from the National Institute of Standards and Technology, Central Institute for Nutrition and Food Research TNO, the on-line computer library (NB) in ITD M/S software program, or previously published literature data.

3.16 Preparation of shrimp extracts for evaluation of antioxidant activity

Shrimp heads, shells and flesh (without the shell) were separated manually from whole shrimp. Extracts from these parts were prepared using either acetone, chloroform, diethyl ether or ethanol. Fifty grams of ground heads or flesh were

homogenized with 100 mL of solvent using a Polytron homogenizer at 1200 rpm for 2 min. However, shells were homogenized with solvent at a ratio of 1:3 (w/v). The slurry obtained was filtered using a Whatman No. 1 filter paper through a Büchner funnel. The acetone, chloroform and diethyl ether extracts were evaporated to dryness using a rotary evaporator and then redissolved in 100 mL of absolute ethanol.

3.17 Determination of total phenolics of shrimp extracts

The content of total phenolics in ethanolic extract of shrimp heads was determined using the Folin-Denis reagent according to Swain and Hillis (1959). Folin-Denis reagent was prepared by combining 100 g of sodium tungstate, 20 g of phosphomolybdic acid and 50 mL of phosphoric acid in 750 mL distilled water. The mixture was refluxed for 2 h, allowed to cool, and then made to 1 L. To 0.1 mL of shrimp ethanolic extract, 0.25 mL Folin-Denis reagent, 0.5 mL saturated sodium carbonate and 2 mL distilled water were added and mixed thoroughly. After 30 min standing at room temperature, mixtures were centrifuged and absorbance values read at 725 nm. Gallic acid was used as a standard (Appendix A2).

3.18 Determination of total carotenoids

Total carotenoids in the lipid fraction of samples were determined according to the method of Saito and Regier (1971). Carotenoids and lipids were extracted from a pre-weighed sample according to Bligh and Dyer (1959). Ten millilitres of the

chloroform layer were evaporated to dryness using a rotary evaporator. The residue was dissolved in 50 mL of petroleum ether. The absorbance of this solution was read at 468 nm. The concentration of carotenoid pigments was calculated based on the following equation:

$$C_{ppm} = A_{468}V_{extract}(\text{dilution factor})/0.2W_{sample}$$

where absorbance of 1 μg/mL of a standard astaxanthin solution at 468 nm was 0.2.

3.19 Evaluation of antioxidant activity using β-carotene-linoleate model system

Antioxidant activities of shrimp extracts or protein hydrolysates (prepared as given in section 3.6) were evaluated using a β-carotene-linoleate model system as described by Miller (1971). A solution of β-carotene was prepared by dissolving 2.0 mg of β-carotene in 10 mL of chloroform. One millilitre of this solution was pipetted into a 50 mL round-bottom flask. Chloroform was then removed under vacuum at 40 °C using a rotary evaporator. Twenty milligrams of purified linoleic acid, 200 mg of Tween 40 emulsifier and 50 mL of aerated distilled water were added to the flask followed by vigorous mixing. Aliquots (5 mL) of the prepared emulsion were transferred to a series of glass tubes containing either 1 mL of shrimp extract, varying amounts of protein hydrolysate, as such, or its fractions on a Sephadex G-15 column. A solution of BHA was used for comparative studies. As soon as the emulsion was added to each tube, the zero time absorbance was read at 470 nm using a HP 8452A diode array spectrophotometer. The solutions were then kept in a water bath at 50°C

and absorbances read at 15 min intervals until the colour of β-carotene was fully bleached (this process took approximately 120 min).

3.20 Chromatographic separation of antioxidative shrimp extract

The isolation of phenolic compounds was carried out by extraction of the crude components from ground shrimp wastes using 95% ethanol (1:2, w/v). The extract was concentrated under reduced pressure using a rotary evaporator at 50 °C, then a stream of nitrogen gas was passed into the vials, sealed and stored at -20 °C until used.

3.20.1 Column chromatography

The separation of the components in the crude extract was carried out by employing column chromatography. A 2.5 cm i.d. x 25 cm column packed with silica gel (60 - 200 mesh, J.T. Baker Chemical Co., Phillipsbury, NJ) was used for this purpose. The column was washed with methanol until the eluted solvent was clear. The column was further washed with approximately 400 mL of n-hexane/acetone (3:1, v/v). Ten grams of the crude ethanolic extract were dissolved in 10 mL ethanol and applied to the column. Two solvent systems were used for elution of the two components with different degrees of polarity. Solvent I was n-hexane/acetone (3:1, v/v) and solvent II was n-butanol/water/acetic acid (3:1:1, v/v/v). Fractions (25 mL each) were collected in 50 mL Erlenmeyer flasks. Each fraction was examined for the types of components present, using thin layer chromatography.

3.20.2 Thin layer chromatography of shrimp isolates

The presence of phenolic compounds in the collected eluate fractions of column chromatography was examined by thin layer chromatography (TLC). TLC plates (Silica Gel 60, with 200 μm thickness, E. Merck Darmstadt, Germany) were developed in a 22 x 22 x 10 cm glass chamber using solvent systems I (n-hexane/acetone, 3:1, v/v) and II (n-butanol/water/acetic acid, 3:1:1, v/v/v) as mobile phases. Plates were sprayed with a ferric chloride reagent (1% ferric chloride in 2.5M HCl), and were placed in a forced-air convection oven (105 °C) for 5 min to allow colour development, then cooled to room temperature. The presence of phenolic compounds is indicated by a brownish yellow coloration.

In another set of experiments, fractions 3-6 separated by solvent I and 6-11 by solvent II were pooled and solvents evaporated. In order to obtain sufficient quantities of components of interest, preparative TLC was employed. The dried isolates from above were dissolved in analytical grade methanol and then applied to Silica gel GF plates (1.0 mm thickness, size: 20 cm x 20 cm; Sigma Chemical Co.). The chromatograms were developed using solvent system I or II as the mobile phase. Compounds from bands with the same R_f values were scrapped off the plate, collected, and then dissolved in methanol. The mixture was centrifuged at 3500 x g for 3 min using an IEC Centra MP4 centrifuge (International Equipment Co.) and the supernatant was evaporated to dryness under a stream of nitrogen.

3.20.3 High performance liquid chromatography of shrimp isolates

The samples obtained by preparative TLC in the preceding section were further purified by semi-preparative HPLC. A Shimadzu chromatographic system (Kyoto, Japan) was used: LC-6A pump, SPD-6AV UV-VIS spectrophotometric detector, SCL-6B system controller and CR 501 chromatopac. Conditions of separation were: the columns used were Particil 10 ODS-2 column (9.4 mm x 250 mm, 10 μm, Whatman) with precolumn (4.6 mm x 125 mm, Whatman) packed with Pellicular ODS (C_{18} groups chemically bonded to 37 - 53 μm glass beads, Whatman); mobile phase 90% (v/v) HPLC-grade methanol (Sigma Chemical Co.) followed by absolute methanol. The flow rate was 3 mL/min and injection volume of sample in methanol was 500 μL. Pure compounds so obtained were also examined by HPLC on an analytical column. An analytical CWSL column (4.5 mm x 250 mm) with spherisorb ODS-2 (10 μm) (Chromatography Sciences Company Inc. Montreal, PQ) was used for this purpose. Flow rate was 1.5 mL/min and injection volume was 20 μL. For both semi-preparative and analytical HPLC, the detector was set at 280 nm. The purity of compounds obtained was also examined by TLC under the above mentioned conditions.

3.20.4 Mass spectrometry

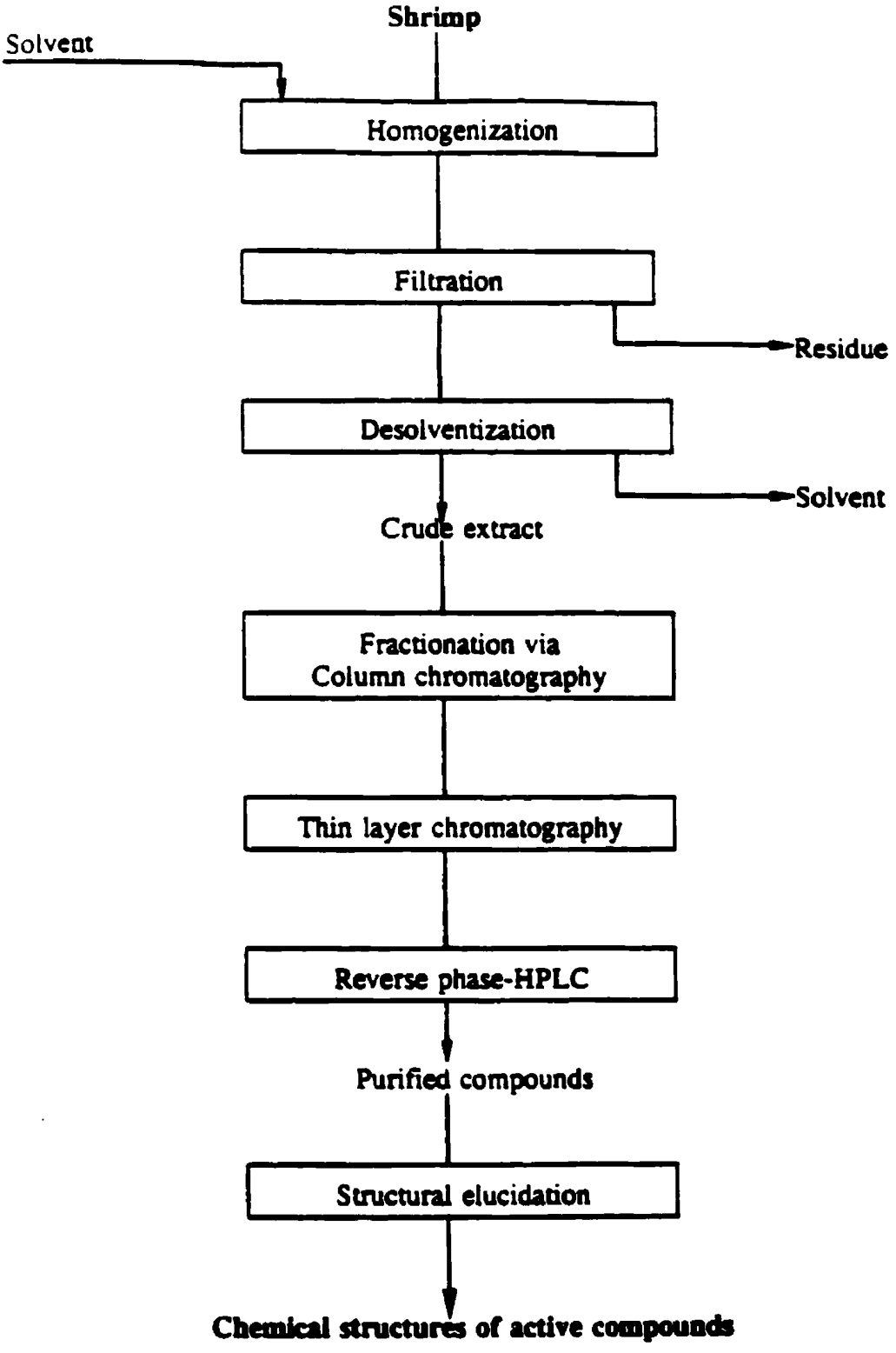
The shrimp isolates purified by reverse-phase HPLC were desolventized and then dissolved in HPLC-grade methanol (approximately 1 mg/mL). A 20 μL aliquot

of the sample was then introduced into the electrospray ion source by continuous flow of acetonitrile/water (70:30, v/v) at a flow rate of 10 μL/mL using a Shimadzu LC-10AD pump connected to the Rheodyne injector with a 20 μL loop. The electrospray (ES) mass spectra (negative-ion mode) were recorded with a Micromass VG-Quattro II quadrupole-hexapole-quadrupole mass spectrometer (Micromass, Cheshire, UK), equipped with an electrospray ionization source and capable of analyzing ions up to *m/z* 4000. A personal computer (Pentium 166 MHz processor) window NT, equipped with Micromass MASSLYNX 3.1 Mass Spectrometry Data System software was used for data acquisition and processing. The temperature of the ES ionization source was maintained at 75 °C. The operating voltage of the ES capillary was 3.0 kV and the high-voltage lens was set at 0.40 kV throughout the whole operation. ES mass spectra were recorded with a cone voltage set to 75 V. Mass spectra were obtained by scanning in the multi-channel analysis (MCA) mode with a scan time of 1 s per 250 amu. Spectra are averages of ten scans. Convectional ES and tandem mass spectra presented in this rationale have been background subtracted and smoothed. A flowsheet for the isolation, purification and structural elucidation of antioxidant compounds in shrimp is given in Figure 3.5.

3.21 Statistical analysis

All experiments in this study were replicated at least three times. Data were reported as mean ± standard deviation (SD). Analysis of variance (ANOVA) was

Figure 3.5 Flowsheet for the isolation, purification and structural elucidation of antioxidant compounds in shrimp.


Shrimp
Solvent
Homogenization
Filtration
Residue
Desolventization
Solvent
Crude extract
Fractionation via
Column chromatography
Thin layer chromatography
Reverse phase-HPLC
Purified compounds
Structural elucidation
Chemical structures of active compounds

performed and differences in mean values determined using Tukey's studentized test at $p < 0.01$ or 0.05 and employing ANOVA and TUKEY'S procedures of statistical analysis system (Snedecor and Cochran, 1980; SAS, 1990). Simple linear and multiple regression analysis were also performed using the same software in the general linear model (GLM) and response surface regression (RSREG) procedures, respectively.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Thermal stable protein dispersions

4.1.1 Proximate composition

The proximate composition of unwashed and washed mechanically separated capelin meat (MSCM) and manually deboned shark meat is given in Table 4.1. The washed meats had a higher moisture and a lower lipid and protein contents as compared with their unwashed counterparts. While approximately an 11.0% increase in moisture content of washed MSCM was noted, a 14.7% moisture increase occurred due to aqueous washing of shark meat. The increased moisture content in the washed meats is due to the hydration of myofibrillar proteins, and this is enhanced by presence of the bicarbonate solution which brings about a slight increase in the pH of the meat, away from the isoelectric pH of myofibrillar proteins (approximately 5.5). A similar observation of elevated moisture in washed products was made by Lee *et al.* (1991) for bicarbonate-washed sardine proteins. The washing process employed a procedure similar to that generally used for preparation of surimi from fatty fish species (Venugopal and Shahidi, 1994). This treatment involves exposure of protein to the moderately high pH of the bicarbonate solution and enhances the unfolding of protein chains, thus influencing their solubility and gelling characteristics (Tanford, 1968; Schmidt, 1981; Howell *et al.*, 1991).

Table 4.1 Proximate composition of unwashed and washed capelin and shark meat[1].

Species/Component	Unwashed (%)	Washed (%)
Capelin		
Moisture	83.98±0.20	93.13±0.10
Crude Protein	12.70±0.31	4.51±0.15
Lipid	1.98±0.01	1.13±0.04
Ash	1.21±0.01	0.42±0.00
Shark		
Moisture	79.81±0.11	91.54±0.11
Crude Protein	18.25±0.32	7.58±0.51
Lipid	1.03±0.05	0.55±0.01
Ash	0.84±0.03	0.13±0.01

[1]Results are mean values of 3 determinations ± standard deviation. Values in the same row are significantly ($p<0.05$) different from one another.

4.1.2 Effect of washing on colour and odour of meat and protein dispersions

The washing process partially removed soluble components of the meat and gave a lighter colour product which was also nearly odourless. For MSCM the Hunter **L** value was increased from 29.9 for unwashed to 34.1 upon washing with water and then a sodium chloride solution. A further increase in Hunter **L** value, to 40.7, was noted when this meat was subsequently washed with a sodium bicarbonate solution. The effect of these washings was also reflected on Hunter **a** and **b** values (for redness and yellowness, respectively), but to a lesser extent as compared with those of the Hunter **L** values (Table 4.2). The lighter colour of the washed MSCM is due to the extraction of pigments which may be present in the starting material. The pigments (myoglobin and haemoglobin) present in mechanically deboned chicken meat have been similarly extracted by washing with water and $NaHCO_3$ solution and resulted in light-coloured meat (Dawson *et al.*, 1988; Shahidi *et al.*, 1992b). There was also extraction of soluble substances from the meats, including metabolic substances such as urea in the case of shark, which is a characteristic of this aquatic species. The original shark meat contained about 780 mg urea per 100 g sample, 92% of which was removed by washing. The characteristic fishy odour was virtually eliminated in the washed meats as noted by the experimenter.

4.1.3 Stability to heat treatment and centrifugation

The resistance to thermal and centrifugal force of unacidified capelin dispersion shows stability of its proteins which were heated at 100 °C followed by centrifugation.

Table 4.2 Hunter **L. a. b** colour values of unwashed and washed mechanically separated capelin meat (MSCM)[1].

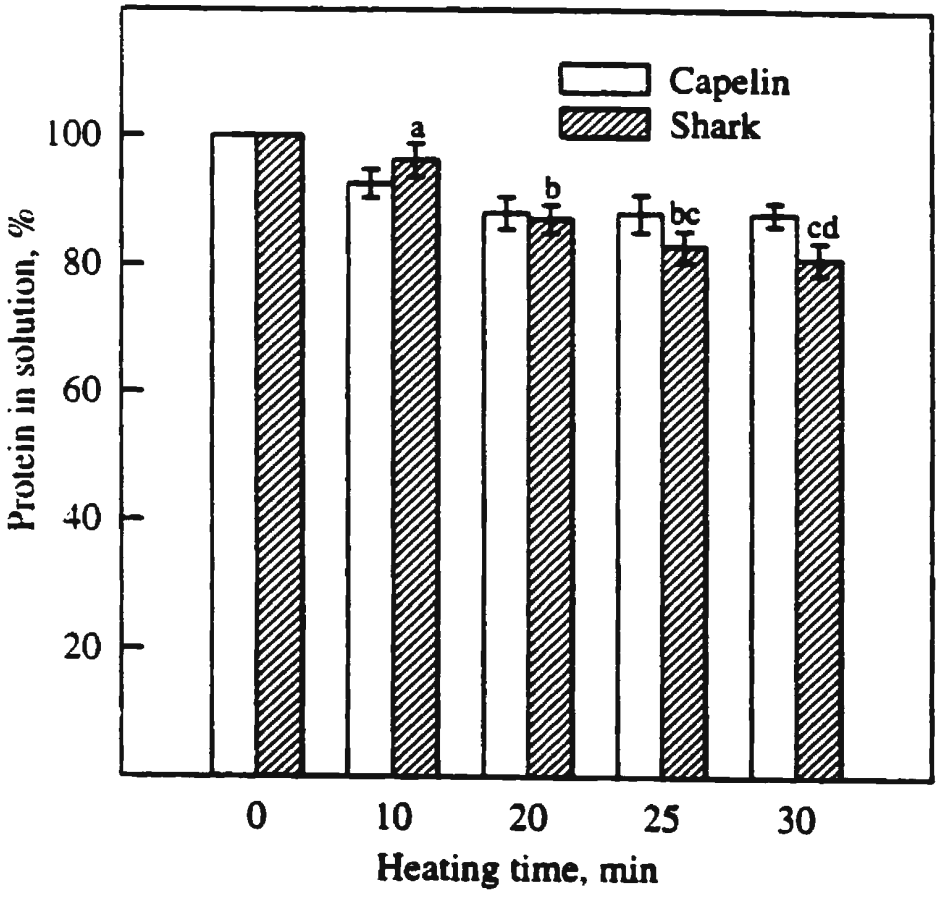
Treatment	Hunter value		
	L	**a**	**b**
Unwashed capelin meat	29.9±0.5^{c}	1.3±0.1^{a}	6.3±0.1^{b}
Washed capelin meat:			
1 x H_2O, then 0.5% NaCl solution	34.1±0.2^{b}	-0.1±0.0^{b}	7.5±0.1^{a}
1 x H_2O, then 0.5% NaCl solution, 0.5% $NaHCO_3$, and finally H_2O	40.7±0.3^{a}	-0.2±0.1^{b}	2.8±0.3^{c}

[1]Results are mean values of 4 determinations ± standard deviations. Values in the same column with the same superscript are not significantly ($p>0.05$) different from one another.

Heat treatment up to 100 °C did not cause precipitation of proteins as over 88% of the original proteins remained soluble in water as dispersion. Heating of aqueous dispersions containing 10-50% (w/v) of washed MSCM at 100 °C followed by centrifugation allowed 88-92.5% of the proteins to remain in water (Figure 4.1). However, heating of unacidified shark meat dispersion prepared by a similar procedure to that used for capelin, at 70 or 100 °C caused coagulation of proteins and hence precipitation in water. This precipitation may have been brought about through enhanced unfolding of proteins, thus making them less compact, more solvated and flexible (Tanford, 1968) which also relates to sodium salt formation of carboxyl groups (Dickinson and Stainsby, 1982). Gelation is a factor that has been suggested to impart thermostability to proteins (Lilliford, 1986; Howell *et al.*, 1991). Gelation of myosin molecules depends upon heating conditions (Sharp and Offer, 1992) and involves irreversible aggregation of myosin heads through formation of disulphide bonds and helix-coil transition of the tail part of the molecules resulting in a three-dimensional network (Niwa, 1992; Stone and Stanley, 1992).

On acidification of shark protein dispersion, using acetic acid, the stability of such proteins to heating at 100 °C for up to 30 min and subsequent centrifugation was demonstrated. As illustrated in Figure 4.1, 87-94% of proteins in the original meat remained in solution after heat treatment for 10 to 30 min followed by centrifugation of the dispersions. The presence of a weak organic acid, i.e. acetic acid, could favour unfolding of the proteins, thus facilitating the gelation process, which in turn enhances the solubility of proteins in water. The relationship between heating condition and gel

Figure 4.1 Effect of heating time at 100°C on protein solubility of capelin and acidified shark protein dispersions. Values having the same letter are not significantly ($p>0.05$) different from one another (shark). All capelin dispersion values are not significantly ($p>0.05$) different from one another.


Capelin
Shark
100
80
60
40
20
Protein in solution, %
a
b
bc
cd
0
10
20
25
30
Heating time, min

strength was explained by Yano (1990), who assumed four different states in the gelation of fish proteins. Thus, the native state of the protein could be changed into a gelation-impossible state which may further be changed to gelation-possible or gelled state, depending upon the environment. It is likely that acetic acid has favoured a gelation-possible state which is converted into a gel by heat treatment.

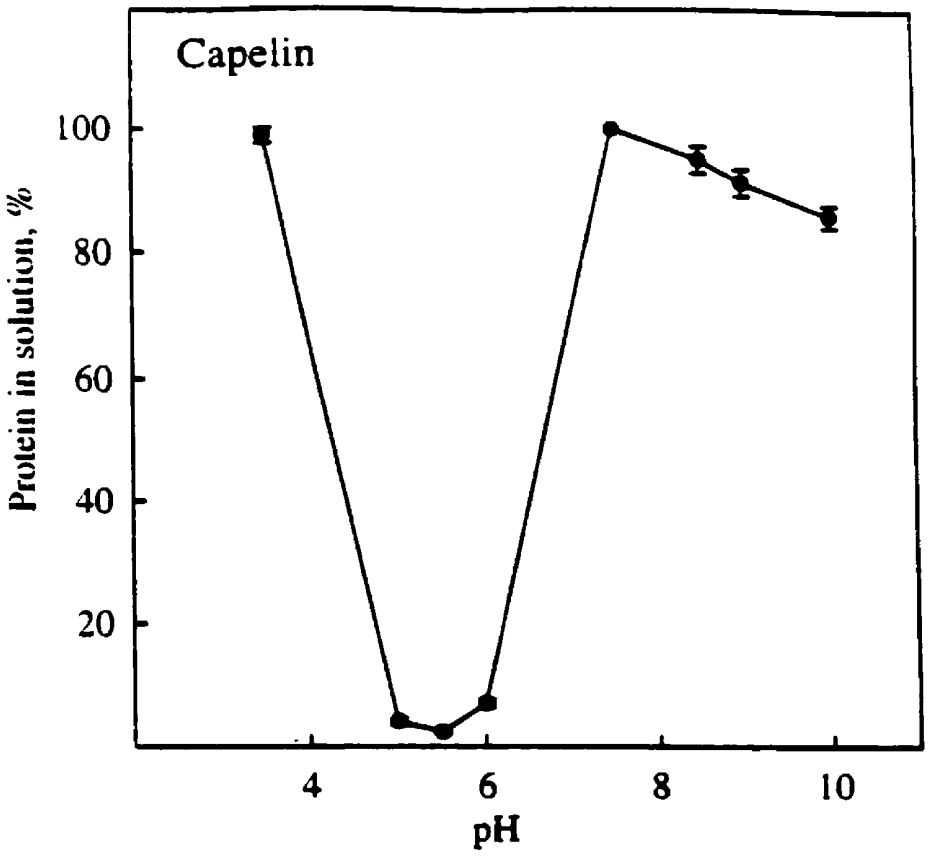
The thermal stability of proteins of aquatic organisms appears to be dependent on a number of factors, some of which are characteristic of a particular species. Although production of thermostable, water dispersions of washed myofibrillar proteins from a number of species has been demonstrated (Shahidi and Venugopal, 1994; Venugopal and Shahidi, 1994), this was not possible for lumpfish proteins. In both unacidified and acidified conditions, these proteins precipitated and remained insoluble in water after heating and subsequent centrifugation. Certain inherent characteristics of lumpfish proteins may be responsible for this observed feature. Lumpfish proteins have a unique morphology which may be responsible for their jelly-like texture and high moisture content. The myofibrillar fraction of muscle proteins consists mainly of myosin, actin and tropomyosin; of these three, myosin is by far the least stable. Howgate and Ahmed (1972) postulated that changes in salt solubility of myofibrillar proteins of cod heated at 30 °C were in fact only due to changes in the myosin component; the actin remaining unchanged and soluble, but unable to be extracted without the myosin. The setting temperature of heated fish paste vary over a range of temperature and this is species-dependent (Hasting *et al.*, 1990). The thermal stability behaviour of lumpfish proteins may possibly be attributed to variations in their myosin component.

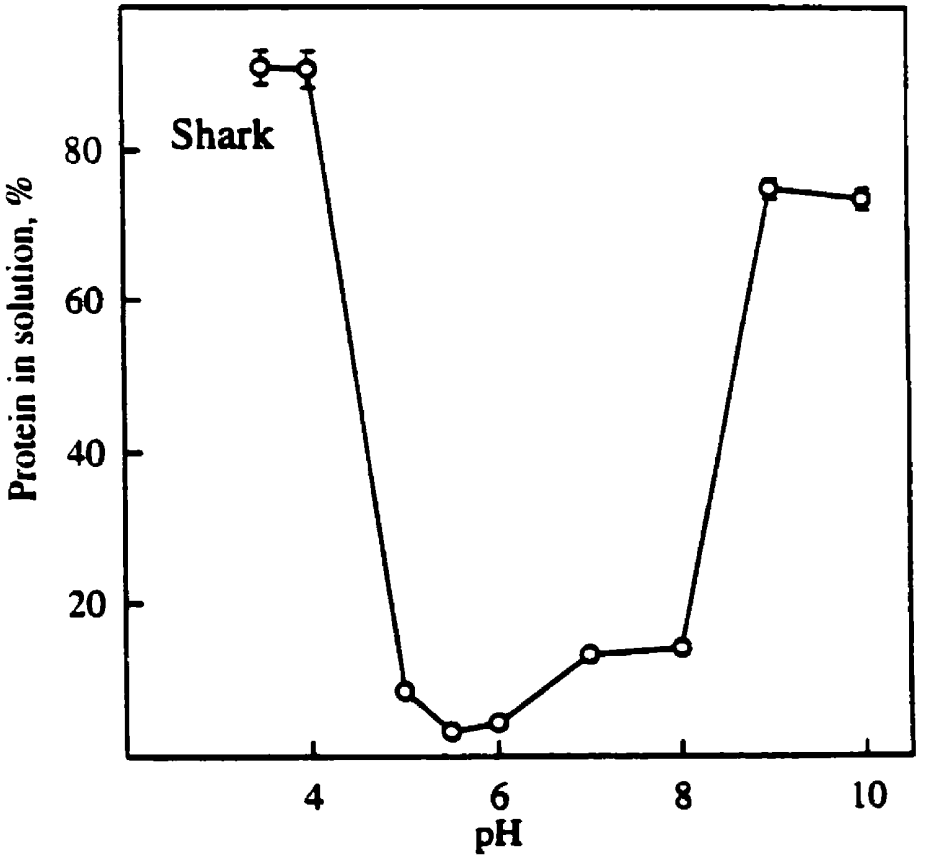
4.1.4 Effect of pH on solubility of capelin and shark protein dispersions

The solubility of protein dispersions of capelin and shark in water was influenced by pH as illustrated in Figure 4.2. In the range of pH studied (3.5 to 10), capelin protein dispersion showed a minimum solubility at pH between 5 and 6, which is in the range of the isoelectric point of main components of myofibrillar proteins. There was increased solubility on both sides of this minimum solubility pH. The proteins, however, remained totally soluble at pH 7.0-7.5. Furthermore, under most pH conditions, over 85% of the proteins remained soluble.

The shark proteins also exhibited very low solubility at pH 5.5-6.0, and a slight increase in solubility up to pH 8.0. Total solubility was achieved at pH 3.5 or lower, and about 75% of proteins remained in solution at pH 9.0-10. Solubility of proteins at different pH values may be explained by ionization of the functional groups of the amino acid residues of proteins in solution, and the overall charges of such proteins depend on the pH of the medium in which they are present. In this case, the net charge of the protein, which is influenced by the pH of the environment, resulted in high solubility, basically by repulsion between the myofibrillar filaments at pH far from isoelectric pH of myofibrillar proteins. However, at pH 5.0-6.0, precipitation of proteins occurred due to low solubility at pH of the environment close to their isoelectric point, when myofibrillar filaments attract each other and become coagulated.

Figure 4.2 Effect of pH variation followed by heating and centrifugation on solubility of proteins from washed capelin (top) and shark (bottom) meats. Error bars were obtained from 3 replicates and where not shown are within the domains of the symbols.


Capelin
Protein in solution, %
100
80
60
40
20
4
6
8
10
pH


Shark
Protein in solution, %
80
60
40
20
4
6
8
10
pH

4.1.5 Effect of protein concentration and temperature on viscosity

The dispersions obtained by homogenization of the washed meat in water were highly viscous, similar to that of other proteins (Tung, 1978). The apparent viscosity of the dispersions depended on the proportion of meat and hence protein content of the dispersion. At a meat content of over 50% (protein content of >2.27%), capelin protein dispersion was too viscous to be effectively homogenized. The apparent viscosity of the unheated capelin dispersion increased with increases in protein concentration of the dispersion. Upon heating of the dispersion to 70 or 100 °C for 15 min, and subsequent cooling to room temperature, the apparent viscosity of the solutions was drastically reduced. Furthermore, while the viscosity of dispersions heated to 70 °C showed some concentration-dependence, the viscosity value of the protein solution was marginal and remained constant at 0.15 Pa.s (Figure 4.3). However, the apparent viscosity of the dispersion may be due to a combination effect related to the size and shape of the protein molecules rather than solely the protein concentration of the dispersion. Viscosity or resistance to flow can be used to monitor protein-protein interaction during heating before a gel is formed (Howell and Lawrie, 1987). Viscosity is also affected by the geometry of proteins, which varies with conformational change (Rha, 1978). A direct correlation between protein concentration and viscosity of unacidified capelin dispersion was obtained (R^2=0.926; Figure 4.4).

Figure 4.3 Effect of protein concentration and heat treatment (70 °C for 15 min) on apparent viscosity of washed capelin protein dispersions. Error bars were obtained from three replicates and where not shown are within the domains of the symbols.

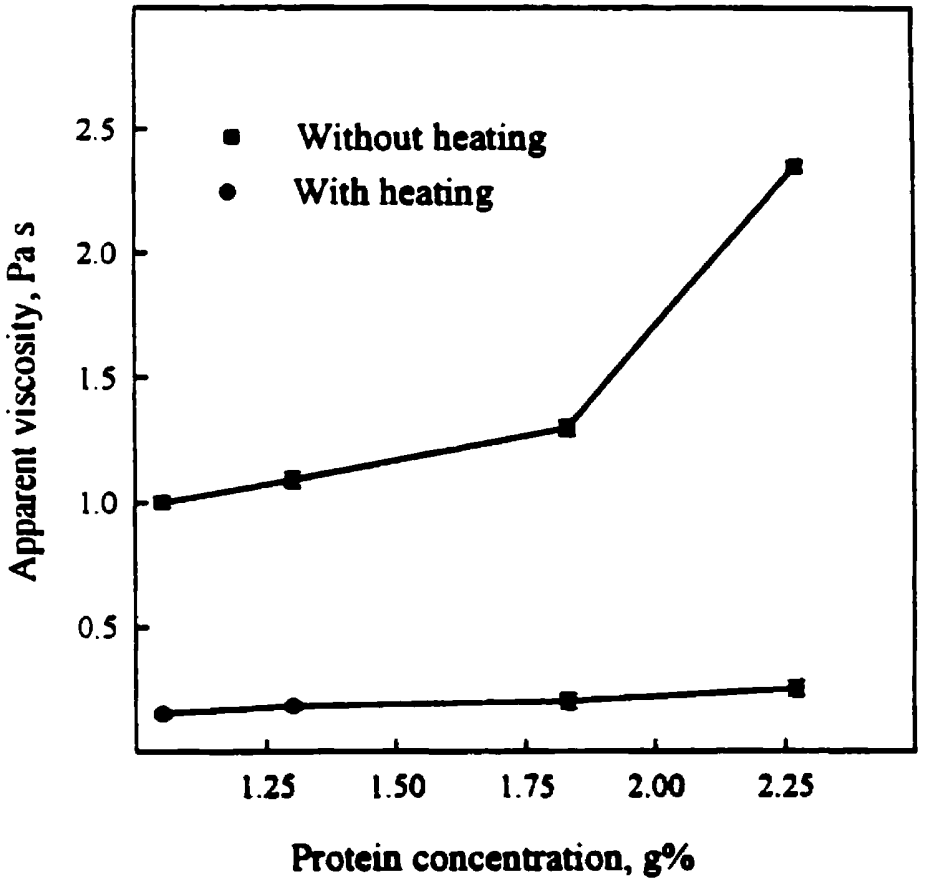
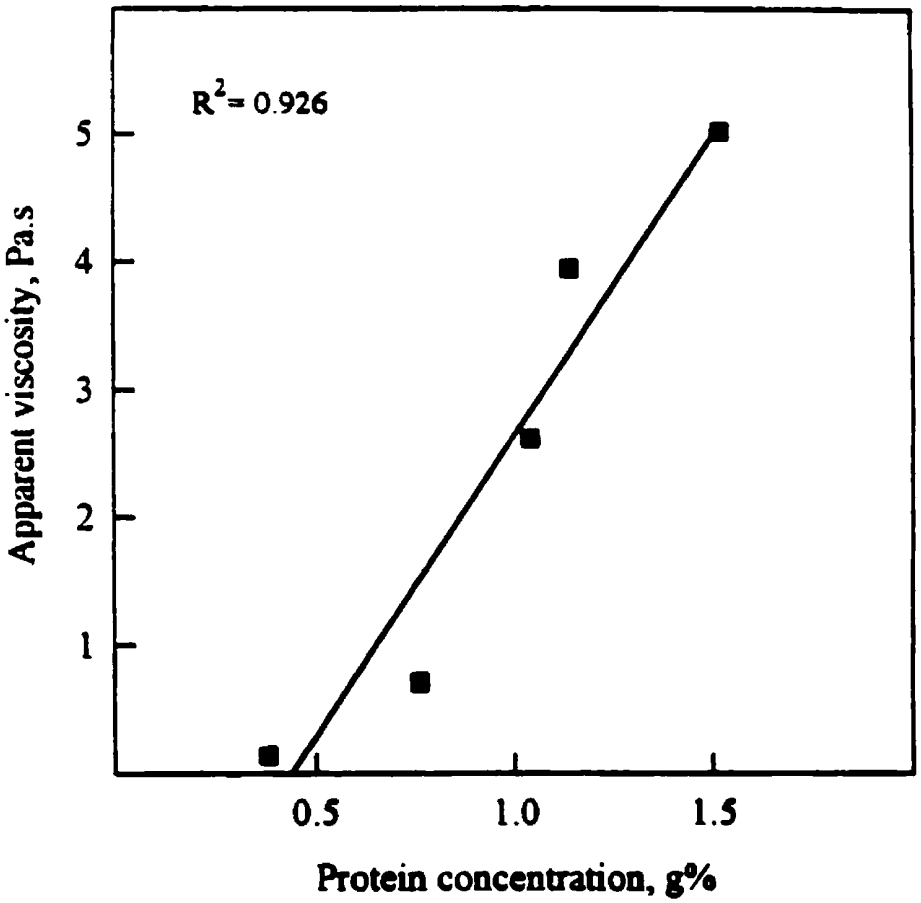

Without heating
With heating
Apparent viscosity, Pa s
2.5
2.0
1.5
1.0
0.5
1.25
1.50
1.75
2.00
2.25
Protein concentration, g%

Figure 4.4 Relationship between protein concentration and viscosity of unacidified shark protein dispersions.


R^2= 0.926
Apparent viscosity, Pa.s
1
2
3
4
5
0.5
1.0
1.5
Protein concentration, g%

The shark protein dispersion exhibited a protein concentration-viscosity relationship, similar to that noted for capelin protein dispersions. The apparent viscosity of the unacidified dispersion, at a meat to water ratio of 1:4 (w/v) having a protein concentration of 0.38%, was 0.14 Pa.s. This viscosity value increased progressively with increasing protein concentration; at a protein concentration of 1.52%, the dispersion had an apparent viscosity of 5.02 Pa.s. However, upon addition of 1 mL acetic acid to 200 mL dispersion, the apparent viscosity dropped drastically and did not exceed 0.11 Pa.s at a protein concentration of 1.52% without affecting the solubility of proteins (Figure 4.5). This could be attributed to low pH-induced conformational changes in the protein molecules (Fink *et al.*, 1990). The acetate ions may bind to the basic amino acids of fish proteins causing changes in their distribution (Sikorski *et al.*, 1990). Furthermore, increased affinity of proteins for water could give a low viscosity, stable protein dispersion.

The unacidified capelin and shark dispersions were also characterized by high apparent viscosities which are dependent upon temperature. Figure 4.6 illustrates the influence of temperature on apparent viscosity of a 20% (w/v) shark meat dispersion (protein content, 1.14%). The apparent viscosity of the preparation decreased with increasing temperature. The viscosity of the unacidified shark protein dispersion was lowered from 5.0 Pa.s at 5°C to 1.52 Pa.s when heated to 45 °C. At higher temperatures, precipitation of proteins occurred. However, the viscosity of the acidified dispersion remained very low (0.04 to 0.20 Pa.s) within the temperature range

Figure 4.5 Effect of protein concentration on apparent viscosity of unacidified and acidified (1 mL acetic acid added to 200 mL dispersion) shark protein dispersions. Error bars were obtained from 3 replicates.

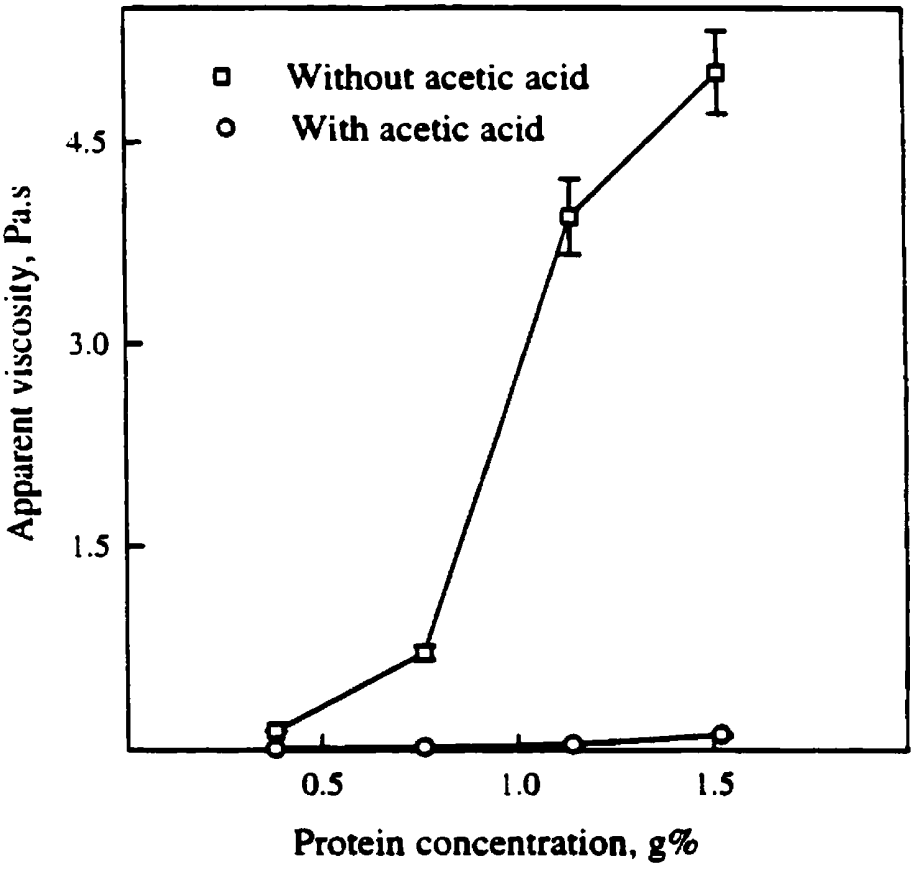
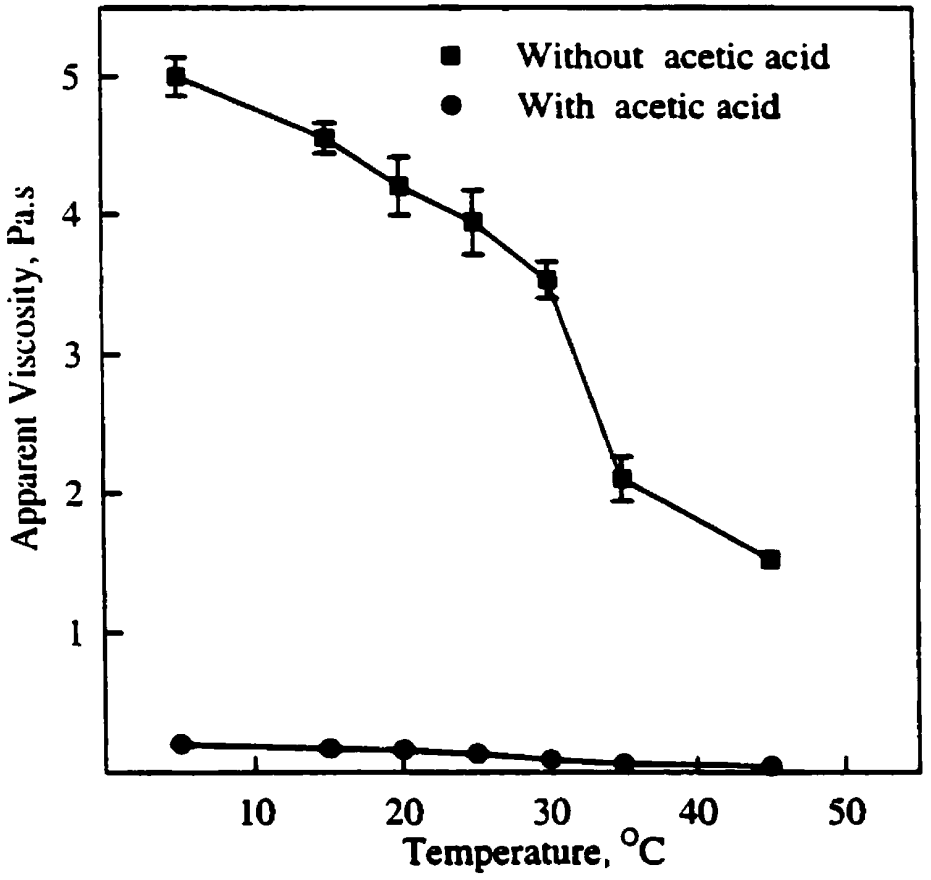

Without acetic acid
With acetic acid
Apparent viscosity, Pa.s
4.5
3.0
1.5
0.5
1.0
1.5
Protein concentration, g%

Figure 4.6 Influence of temperature on apparent viscosity of unacidified and acidified shark protein dispersions. Error bars were obtained from 3 replicates and where not shown are within the domains of the symbols.


Without acetic acid
With acetic acid
Apparent Viscosity, Pa.s
5
4
3
2
1
10
20
30
40
50
Temperature, °C

(5 to 45 °C) studied. Heating the acidified dispersion for up to 30 min at 100 °C did not have any pronounced effect on viscosity of the dispersion, due to the already low viscosity values obtained. The unacidified shark dispersion had an initial viscosity of 3.94 Pa.s and there was approximately a 95% decrease in this value when the dispersion was heated for 30 min (Figure 4.7). This behaviour could be attributed to heat-induced redistribution of weak forces such as hydrogen bonds, electrostatic and steric interactions leading to conformational changes in the proteins (Hermansson *et al.*, 1986). Heating may also degrade the α-helical structure of proteins in surimi paste, a portion of which is recovered upon cooling. Moreover, heating enhances the hydrophobicity of myofibrillar proteins (Niwa, 1992).

4.1.6 Effect of salt on thermal stability of proteins

The influence of addition of NaCl, KCl or $CaCl_2$, with or without sodium tripolyphosphate (STPP), in combination with heat, on stability of capelin protein dispersions is shown in Figure 4.8. The presence of mono- and divalent cation chlorides exhibited varying degrees of solubility effects on capelin proteins. While proteins remained fairly soluble in 50mM NaCl or KCl solution, the presence of $CaCl_2$ resulted in precipitation of 72-74% of proteins. STPP, together with these salts, generally enhanced the solubility of proteins. This effect may be due to the ability of STPP to increase the pH and to dissociate actomyosin into actin and myosin (Hamm, 1970). The effect of salts may be explained by their ability to diminish

Figure 4.7 Effect of heating time at 100 °C on apparent viscosity of unacidified and acidified shark protein dispersions. Error bars were obtained from 3 replicates.

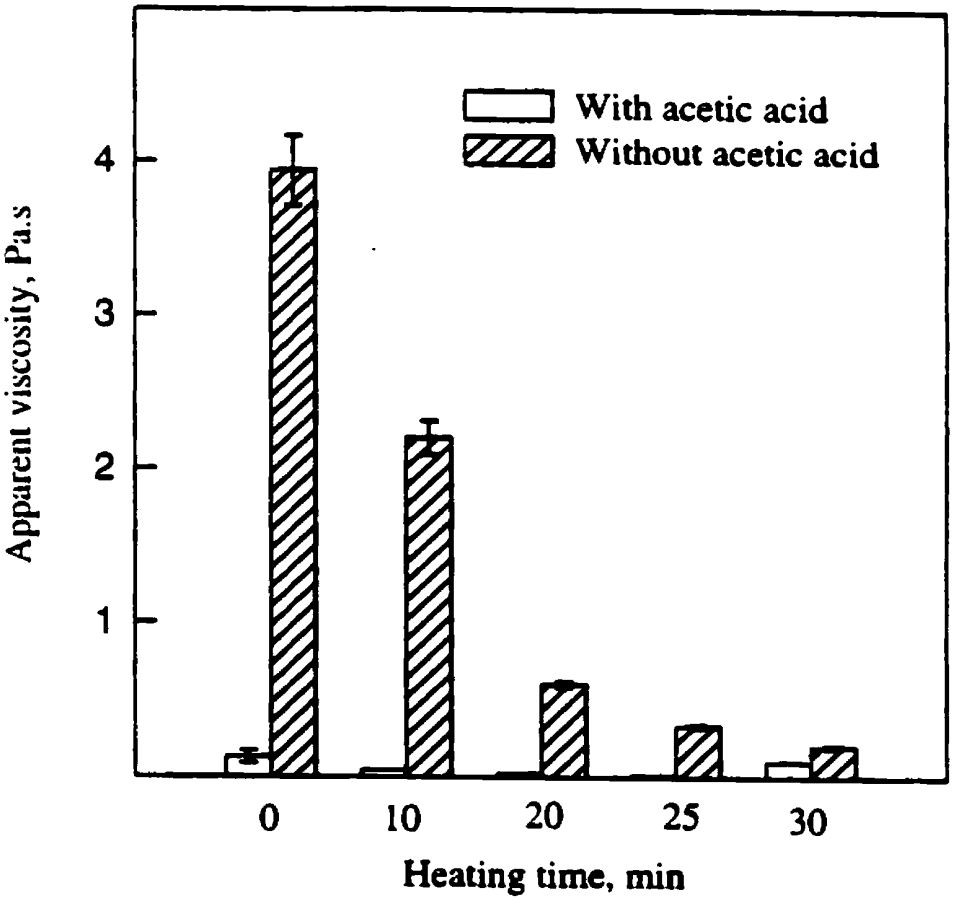
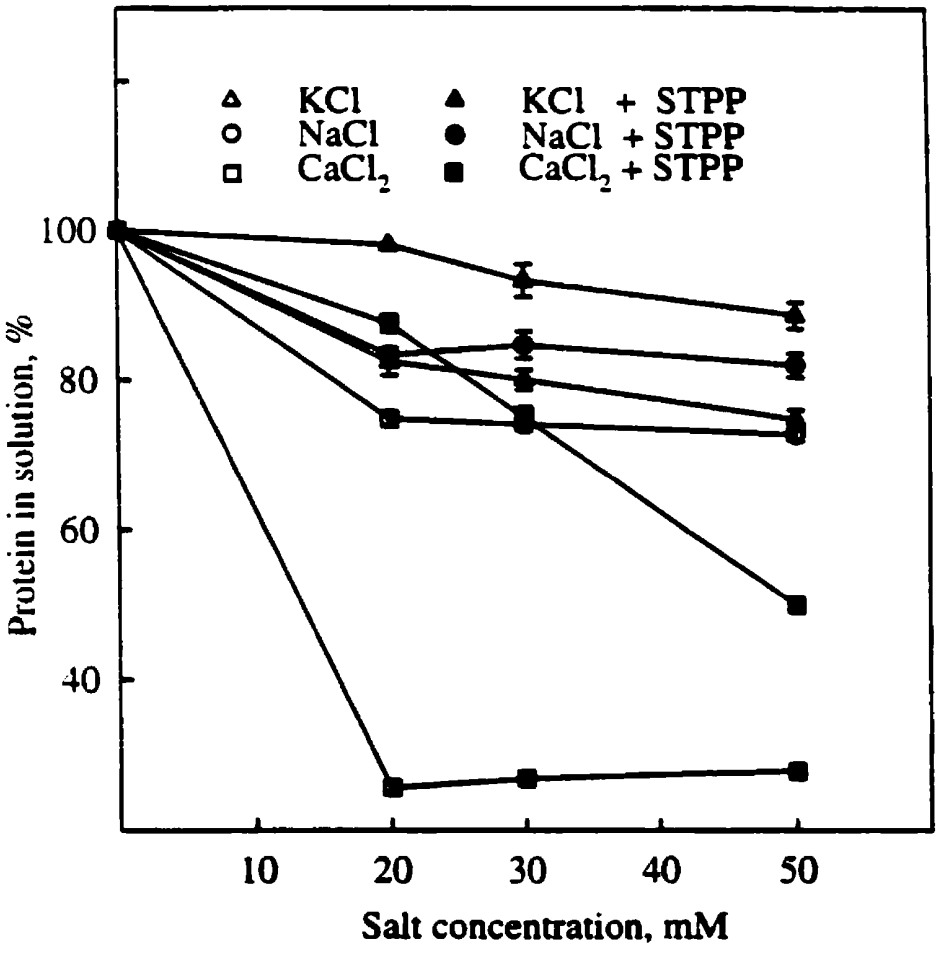

With acetic acid
Without acetic acid
Apparent viscosity, Pa.s
4
3
2
1
0
10
20
25
30
Heating time, min

Figure 4.8 Effect of presence of KCl, NaCl and Ca_2Cl with or without sodium tripolyphosphate (STPP) on solubility of proteins from washed capelin meat. Error bars were obtained from 3 replicates.


KCl
NaCl
CaCl2
KCl + STPP
NaCl + STPP
CaCl2 + STPP
100
80
60
40
Protein in solution, %
10
20
30
40
50
Salt concentration, mM

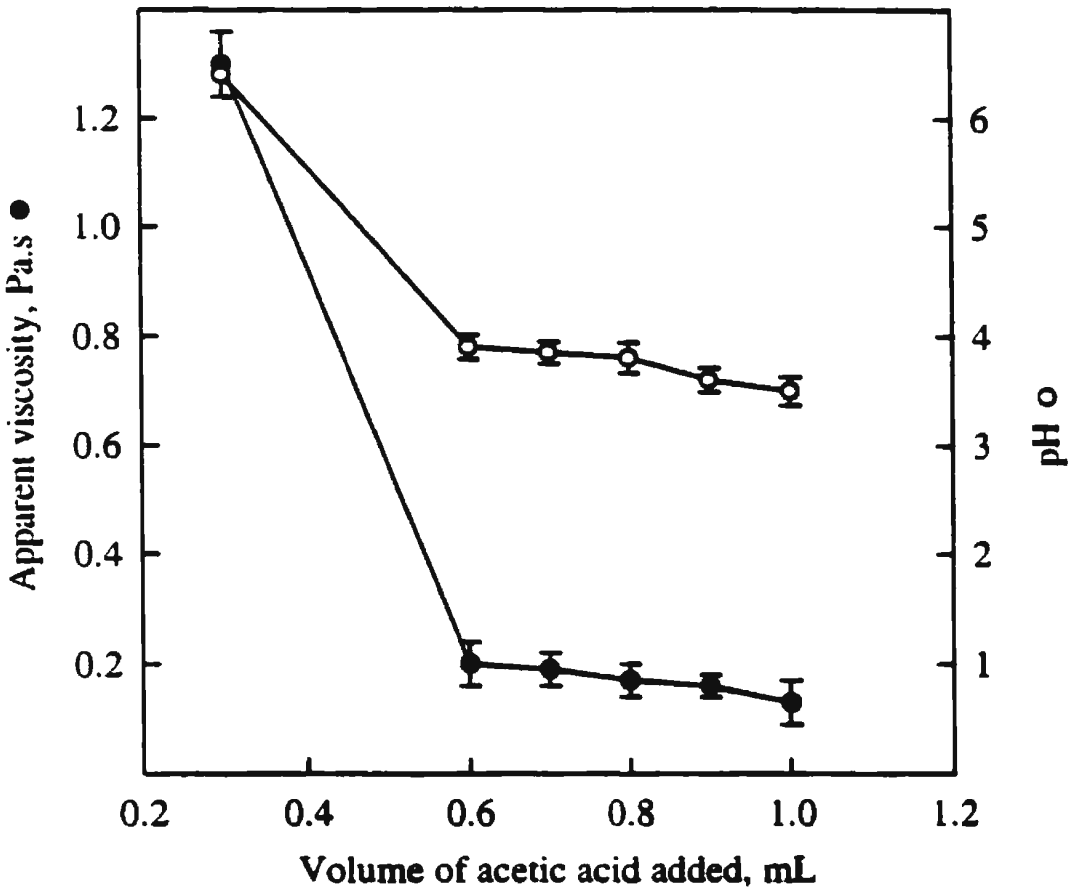
electrostatic repulsive forces and increase the hydrophobic properties of protein molecules, thus leading to aggregation and precipitation. The effects of individual salts in this regard, appears to be concentration dependent. However, $CaCl_2$ was more effective in inducing aggregation and precipitation, probably due to its ability to form bridges between charged groups (Foegeding, 1988). The results of the effects of salts on thermal stability of proteins in this study are in accord with those of Hermansson *et al.* (1986) who reported partial separation of water from protein gels in the presence of 5% salt.

4.1.7 Effect of concentration of acetic acid and pH on viscosity of dispersions

The addition of acetic acid, up to 1% (w/v), to shark homogenate drastically decreased its apparent viscosity (Figure 4.9). The incorporation of 0.4 mL of acetic acid into 200 mL of the dispersion afforded a homogenate with an apparent viscosity of 1.3 Pa.s. However, further addition of 0.2 mL of acid resulted in a sharp drop in viscosity to 0.2 Pa.s; further addition of acetic acid, up to 1 mL, to the homogenate had a slight additional effect on its apparent viscosity. This indicated that the critical point for decreased apparent viscosity was reached when 0.6 mL acetic acid was added to 200 mL of dispersion. The concentration of acetic acid in the dispersion appears to have a drastic effect on decreasing the apparent viscosity to very low values.

The pH of the dispersion also influenced its apparent viscosity. The initial pH of the dispersion was 7.1 in the absence of acetic acid. On addition of 0.3 mL acetic

Figure 4.9 Influence of acetic acid concentration on pH and apparent viscosity of washed shark protein dispersion. Error bars were obtained from 3 replicates.


Apparent viscosity, Pa.s ●
pH o
Volume of acetic acid added, mL
1.2
1.0
0.8
0.6
0.4
0.2
6
5
4
3
2
1
0.2
0.4
0.6
0.8
1.0
1.2

acid the pH was lowered to 6.5 and then to 3.5 when 0.3 mL more acetic acid was added. There was a drop in apparent viscosity from 1.3 Pa.s at pH 6.5 to 0.8 Pa.s at pH 3.5.

4.1.8 Total and free amino acid content and protein efficiency ratio (PER) of unwashed and washed muscle proteins of capelin and shark

Table 4.3 shows the total amino acid profile of unwashed and washed capelin and shark meats. The content of individual amino acids of washed and original meats of capelin was similar, indicating that the extraction process did not have any marked effect on the amino acid profile of samples. A similar trend in amino acid composition was observed for shark protein. This is an indication that there was no preferential loss of any particular amino acid due to aqueous washings. Aqueous washing of mackerel and resultant dispersions prepared from it, had a comparable amino acid composition to that of the whole meat (Venugopal and Shahidi, 1994). The largest change (approximately 7.6 and 8.3%, respectively) was noted for glycine and proline contents of shark meats, respectively, presumably due to the concentration of connective tissues in the washed meats, as these amino acids are prevalent in the connective tissues (Lee *et al.*, 1978). The calculated protein efficiency ratio (PER) values obtained, using equations proposed by Lee *et al.* (1978), indicated no adverse effect due to aqueous washings on the nutritional value of products. The PER values of washed capelin and shark meats were 3.09-3.26 and 3.00-3.22, respectively, as compared with 3.09-3.25 and 3.09-3.19 for their unwashed counterparts (Table 4.4).

Table 4.3 Total amino acid composition of unwashed and washed capelin and shark meats (g/100g protein)[1].

Amino acid	Unwashed capelin	Washed capelin	Unwashed shark	Washed shark
Alanine	6.08±0.06	6.11±0.08	5.89±0.26	5.77±0.17
Arginine	6.33±0.12	6.53±0.20	6.70±0.36	6.61±0.08
Aspartic acid[2]	10.88±0.10	10.87±0.10	9.43±0.37	9.63±0.21
Cysteine	0.93±0.02	0.90±0.03	1.27±0.08	1.89±0.09
Glutamic acid[3]	14.86±0.12	14.64±0.16	13.90±0.25	13.93±0.38
Glycine	5.33±0.12*	5.74±0.25*	4.76±0.20	4.87±0.12
Histidine	2.35±0.05	2.18±0.03	2.49±0.07	2.31±0.02
Hydroxyproline	0.21±0.02*	0.11±0.01*	0.44±0.04*	0.77±0.03*
Isoleucine	4.72±0.18	4.88±0.10	5.65±0.23	5.54±0.11
Leucine	9.19±0.16	9.25±0.20	7.64±0.11	7.70±0.20
Lysine	9.54±0.08	9.47±0.05	9.16±0.33	8.91±0.11
Methionine	3.08±0.10	3.11±0.09	3.52±0.09	3.60±0.06
Phenylalanine	4.42±0.10	4.20±0.15	4.30±0.16	4.14±0.05
Proline	3.63±0.05*	3.93±0.05*	3.57±0.17*	3.97±0.06*
Serine	4.41±0.10	4.26±0.18	4.02±0.23	3.65±0.08
Threonine	4.83±0.12	4.95±0.10	4.85±0.20	4.61±0.08
Tryptophan	1.08±0.10	0.99±0.10	1.21±0.08	1.09±0.05
Tyrosine	4.00±0.08	4.11±0.05	3.72±0.14	3.61±0.05
Valine	5.72±0.10	5.78±0.10	5.68±0.24	5.37±0.08

[1]Results are mean values of 3 replicates ± standard deviation. Values in each row with * (for each species) are significantly ($P<0.05$) different from one another.

[2]Aspartic acid and asparagine.

[3]Glutamic acid and glutamine.

Table 4.4 Protein efficiency ratio (PER) values of unwashed and washed capelin meats and their comparison with those of cod proteins.

Equations[1]	Unwashed capelin	Washed capelin	Unwashed shark	Washed shark	cod[2]
1	3.25	3.26	3.19	3.22	2.99
2	3.09	3.09	3.09	3.00	2.91

[1]See the equations in Section 3.8.1 of Material and Methods.
[2]From Shahidi *et al.* (1991).

In contrast, the total contents of free amino acids in washed capelin and shark meats were reduced by about 92 and 87%, respectively (Table 4.5). Free amino acids are highly water-soluble and are easily removed by the washing process (Onodenalore, 1993). The extraction of free amino acids afforded a bland product; such preparations may be used in formulations of a variety of products and where possible addition of flavourants may be considered.

4.2 Preparation of protein hydrolysates

4.2.1 Progress curves for enzymatic hydrolysis

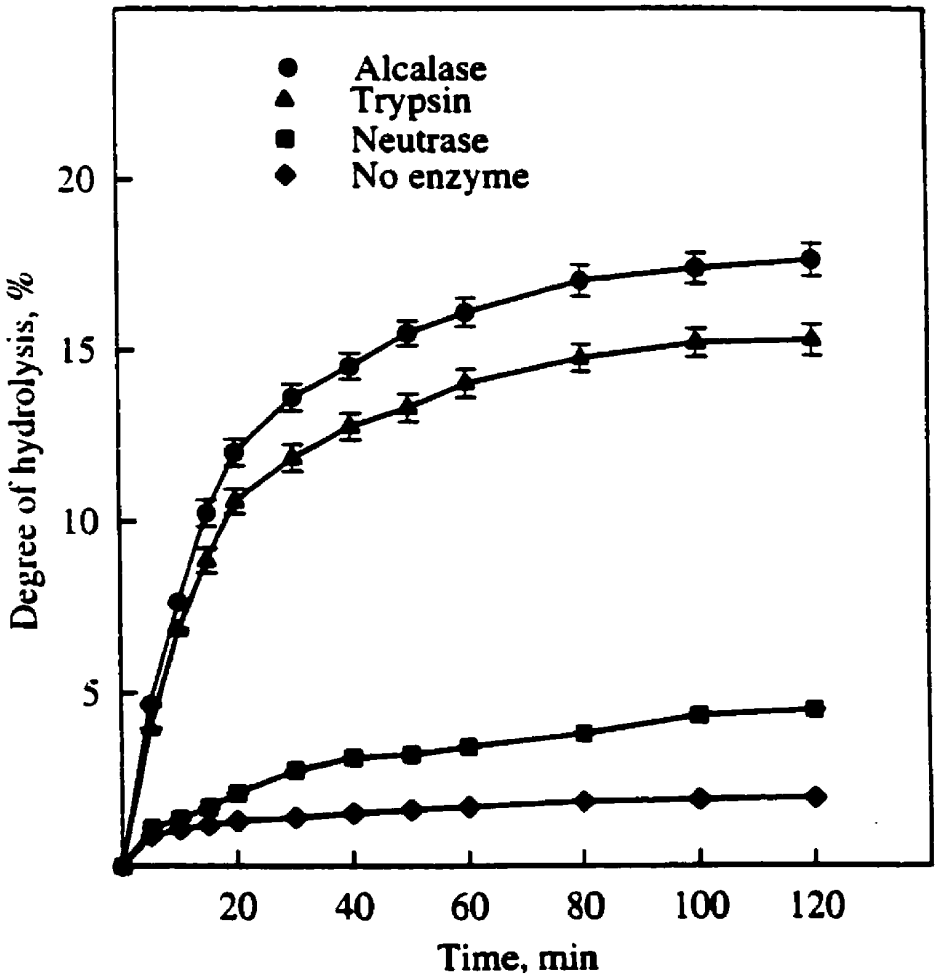
Reaction progress curves for enzymatic hydrolysis of shrimp heads with and without Alcalase, trypsin and Neutrase during a 2-h hydrolysis period were compared (Figure 4.10). A typical triphasic hydrolysis curve was obtained, exhibiting a fast reaction rate followed by a slowing, and eventually a flattening of the curve, where no further hydrolysis seems to occur. Similar progress curves have been reported for enzymatic hydrolysis of fish proteins (Archer *et al.*, 1973), soybean (Adler-Nissen, 1979), lean beef tissue (O'Meara and Munro, 1984), casein (Mannheim and Cheryan, 1990), crayfish processing by-products (Baek and Cadwallader, 1995) and capelin (Shahidi *et al.*, 1995). Trypsin and Neutrase showed a similar trend to that observed for Alcalase, however, lower degrees of hydrolysis were exhibited, perhaps due to lower enzyme activities. In the absence of enzymes, a very marginal degree of hydrolysis was noted giving a curve that runs close to the x-axis (Figure 4.10).

Table 4.5 The content of free amino acids of unwashed and washed capelin and shark meats (mg/100g sample)[1].

Amino acid	Unwashed capelin	Washed capelin	Unwashed shark	Washed shark
Alanine	44.8±0.6	3.3±0.2	14.5±2.33	1.71±0.05
Arginine	11.7±0.3	3.5±0.1	1.09±0.14	0.00±0.00
Aspartic acid	22.7±1.0	2.3±0.1	4.29±0.07	0.51±0.02
Cysteine	2.3±0.1	trace	0.35±0.02	0.19±0.00
Glutamic acid	43.6±1.3	2.8±0.3	8.95±0.14	1.16±0.04
Glutamine	15.1±0.4	3.8±0.1	6.84±0.03	0.65±0.01
Glycine	22.1±0.6	1.4±0.1	6.89±0.21	0.78±0.02
Histidine	10.0±0.3	0.8±0.1	2.29±0.03	0.49±0.01
Isoleucine	19.5±0.5	1.6±0.2	2.61±0.01	0.37±0.01
Leucine	37.4±0.8	3.0±0.1	5.79±0.04	0.75±0.02
Lysine	39.3±0.9	10.2±0.1	5.26±0.03	0.80±0.04
Methionine	14.0±0.6	1.5±0.2	3.55±0.01	0.82±0.04
Phenylalanine	17.7±0.5	2.4±0.3	3.49±0.09	0.66±0.03
Proline	22.6±0.2	1.3±0.2	5.65±1.36	0.29±0.00
Serine	26.9±0.8	2.7±0.5	7.44±0.12	0.95±0.04
Taurine	129.6±2.3	4.1±0.6	32.0±0.42	2.47±0.08
Threonine	22.3±0.1	1.6±0.1	5.53±0.02	0.66±0.03
Tryptophan	3.1±0.3	0.2±0.1	0.53±0.03	0.90±0.04
Tyrosine	16.6±0.2	2.1±0.1	1.72±0.01	0.32±0.01
Valine	31.1±0.5	2.3±0.3	4.22±0.27	0.60±0.02
Total	**618.5**	**50.9**	**123**	**16.0**

[1]Results are mean values of 3 replicates ± standard deviation. Values in each row (for each species) are significantly ($p<0.05$) different from one another.

Figure 4.10 Typical hydrolysis curves of enzyme-assisted shrimp protein hydrolysis involving Alcalase, Trypsin and Neutrase. Error bars were obtained from results of 3 replicates.


Alcalase
Trypsin
Neutrase
No enzyme
Degree of hydrolysis, %
20
15
10
5
20
40
60
80
100
120
Time, min

There are many possible explanations for the progress curve obtained. Adler-Nissen (1986a) attributed the shape of a progress curve to substrate competition (a kind of product inhibition) between the original substrate and peptides produced during hydrolysis. O'Meara and Munro (1984) concluded that this type of curve was the result of rapid cleavage of susceptible peptide bonds during the initial stages and slow cleavage of the less susceptible bonds at later stages. In enzymatic hydrolysis of an insoluble substrate, Archer *et al.* (1973) suggested that enzymes adsorbed onto an insoluble protein particle in a fast reaction, degraded polypeptides chains that were loosely bound to the surface, and acted on the more compact core protein slowly. A typical triphasic type of hydrolysis curve obtained in our study may be due to one or any combination of these factors. A downward curvature of progress curves was reported in the enzymatic hydrolysis of crayfish processing by-products, and was also attributed to a combination of the above factors (Baek and Cadwallader, 1995).

4.2.2 Optimization of hydrolysis conditions for preparation of shrimp and lumpfish hydrolysates

The experiments involving screening on the three enzymes considered in this study indicated that Alcalase had the highest efficiency, under the hydrolysis conditions examined, in producing the desired degree of hydrolysis (DH) and nitrogen recovery (NR) in the preparation of both shrimp and lumpfish protein hydrolysates. However, there are many factors that affect the DH and yield of product during

enzymatic hydrolysis of proteins, and these include temperature, pH and enzyme/substrate concentration ratio, among others (Adler-Nissen, 1986b). It was therefore prudent to study these factors collectively in order to find the optimum reaction conditions to obtain a maximum degree of hydrolysis and nitrogen recovery in Alcalase-assisted hydrolysis of shrimp and lumpfish proteins. The variables considered for the hydrolysis were temperature (X_1), pH (X_2) and enzyme/substrate concentration ratio (X_3). Enzyme concentration is a major factor that affects the production of protein hydrolysate by enzymatic means and also influences the production cost (Baek and Cadwallader, 1995). However, reaction conditions such as pH and temperature of the medium also play important roles in creating the most favourable conditions for maximum enzyme activity and hence the desired optimum product properties. The preliminary experiments indicated that a suitable range of enzyme/substrate concentration ratio for optimization experiments to be 20 - 24 and 15 - 35 AU/kg protein for shrimp and lumpfish, respectively. The differences between these two protein sources may be due to compositional and sequence differences which affected the rate of hydrolysis and hence the amount of enzyme required. Furthermore, presence of shells may also affect the rate of hydrolysis of shrimp proteins, although no studies have been carried out to confirm this possibility.

4.2.3 Diagnostic checking and fitted models

Tables 4.6 and 4.7 give the degrees of hydrolysis as well as nitrogen recovery for shrimp and lumpfish hydrolysates, respectively. For both responses, higher values

Table 4.6 Cube-centred design arrangement and responses for enzymatic hydrolysis experiments of shrimp proteins.[1]

Run	Variable levels			Responses (Y)[3]	
	Temp, °C (X_1)	pH (X_2)	Enz/subs[2] conc (X_3)	Degree of hydrolysis, %	Nitrogen recovery, %
1	45	7	20	11.4	48.1
2	45	7	40	11.9	51.6
3	45	9	20	11.8	50.5
4	45	9	40	17.2	76.9
5	65	7	20	13.8	67.5
6	65	7	40	15.7	70.6
7	65	9	20	12.2	64.9
8	65	9	40	12.9	66.0
9	45	8	30	12.7	65.1
10	65	8	30	15.8	72.1
11	55	7	30	13.6	66.9
12	55	9	30	12.5	64.1
13	55	8	20	13.1	65.8
14	55	8	40	17.6	77.4
15	55	8	30	17.1	77.1
16	55	8	30	17.8	77.6
17	55	8	30	18.1	78.1
18	55	8	30	18.3	78.4

[1] Nonrandomized
[2] Enzyme/substrate concentration ratio, AU/kg protein
[3] Average value of duplicate determinations except for Runs 15 to 18.

Table 4.7 Cube-centred design arrangement and responses for enzymatic hydrolysis experiments of lumpfish proteins.[1]

Run	Variable levels			Responses (Y)[3]	
	Temp, °C (X_1)	pH (X_2)	Enz/subs[2] conc (X_3)	Degree of hydrolysis, %	Nitrogen recovery, %
1	45	7	15	12.6	58.4
2	45	7	35	15.8	69.5
3	45	9	15	12.9	53.1
4	45	9	35	15.3	64.3
5	65	7	15	14.9	67.5
6	65	7	35	16.9	73.6
7	65	9	15	15.9	65.8
8	65	9	35	16.9	73.7
9	45	8	25	16.1	68.7
10	65	8	25	17.7	74.3
11	55	7	25	15.8	70.4
12	55	9	25	16.8	70.2
13	55	8	15	15.2	63.8
14	55	8	35	17.4	78.0
15	55	8	25	18.2	80.2
16	55	8	25	17.9	79.9
17	55	8	25	18.2	78.9
18	55	8	25	18.6	79.1

[1] Nonrandomized
[2] Enzyme/substrate concentration ratio, AU/kg protein
[3] Average value of duplicate determinations except for Runs 15 to 18.

were obtained for lumpfish hydrolysates than for their shrimp counterparts. The data obtained from eighteen experimental points were used for statistical analysis to optimize the process variables (temperature, pH and enzyme/substrate concentration ratio) in the preparation of lumpfish and shrimp protein hydrolysates. Multiple linear regression coefficients, obtained using the least squares method, were used to predict quadratic polynomial models for the degree of hydrolysis and nitrogen recovery for shrimp and lumpfish protein hydrolysates and are summarised in Tables 4.8 and 4.9, respectively.

The analysis of variance (ANOVA) for regression was performed to assess the degree of fitness of the quadratic model used in this experiment. The coefficients of independent variables, temperature (X_1), pH (X_2) and enzyme/substrate concentration ratio (X_3) determined for quadratic polynomial models for degree of hydrolysis (Y_1) and nitrogen recovery (Y_2) of the prepared shrimp protein hydrolysates were:

$$Y_1 = -224.6688 + 3.5808X_1 + 34.1175X_2 + 0.2143X_3 - 0.0293X_1^2 - 2.0300X_2^2 - 0.1815X_3^2 - 0.0650X_1X_2 + 0.0078X_1X_3 + 0.0675X_2X_3.$$

$$Y_2 = -724.3625 + 15.8810X_1 + 17.6850X_2\ 2.7410X_3 - 0.1141X_1^2 - 3.4600X_2^2 - 0.0711X_3^2 - 0.3750X_1X_2 + 0.0120X_1X_3 + 0.1350X_2X_3.$$

Table 4.8 Regression coefficients of predicted quadratic polynomial model for two response variables (degree of hydrolysis and nitrogen recovery) of shrimp hydrolysis.

Coefficient[1]	Degree of hydrolysis, % (Y_1)	Standard error of Y_1	% Nitrogen recovery (Y_2)	Standard error of Y_2
Intercept (β_0)	-224.66875***	26.7245	-724.3625***	137.3107
Linear				
β_1	3.58075***	0.4290	15.8810***	2.2042
β_2	34.11750***	5.2567	73.6850**	27.0089
β_3	0.21425	0.3549	2.7410	1.8233
Quadratic				
β_{11}	-0.02930***	0.0031	-0.1141***	0.0157
β_{22}	-2.03000***	0.3046	-3.4600	1.5650
β_{33}	-0.18150***	0.0003	-0.0711**	0.0157
Interaction				
β_{12}	-0.06500	0.0313	-0.3750	0.1606
β_{13}	0.00775**	0.0031	0.0120	0.0161
β_{23}	0.06750	0.0313	0.1350	0.1606
β_{123}	-	-	-	-
R^2	0.9714		0.9508	
Probability>F	0.0001		0.0009	
CV %	4.3475		4.7875	

[1]See model given in Section 3.2.5.
** $p<0.05$
*** $p<0.01$

Table 4.9 Regression coefficients of predicted quadratic polynomial model for two response variables (degree of hydrolysis and nitrogen recovery) of lumpfish hydrolysis.

Coefficients[1]	Degree of hydrolysis, % (Y_1)	Standard error of Y_1	% Nitrogen recovery (Y_2)	Standard error of Y_2
Intercept (β_0)	-88.87625***	20.6684	-332.90694**	119.2652
Linear				
β_1	0.63475	0.3883	3.54064	2.2410
β_2	17.91750***	5.2845	65.98131**	30.4961
β_3	1.02925***	0.2438	2.89574	1.4072
Quadratic				
β_{11}	-0.00525	0.0032	-0.03420	0.0186
β_{22}	-1.12500***	0.3224	-4.62024**	1.8606
β_{33}	-0.01125***	0.0032	-0.04020	0.0186
Interaction				
β_{12}	0.01500	0.0188	0.11125	0.1083
β_{13}	-0.00325	0.0019	-0.01037**	0.0108
β_{23}	-0.02250	0.0188	0.02375	0.1083
β_{123}	-	-	-	-
R^2	0.9543		0.9238	
Probability>F	0.0002		0.0014	
CV %	3.2594		4.3431	

[1]See model given in Section 3.2.5.
** $p<0.05$
*** $p<0.01$

Meanwhile, the quadratic polynomial models for Y_1 and Y_2 for the prepared lumpfish protein hydrolysates were:

$$Y_1 = -88.8762 + 0.6348X_1 + 17.9175X_2 + 1.0292X_3 - 0.0053X_1^2 - 1.1250X_2^2 - 0.0113X_3^2 + 0.0150X_1X_2 - 0.0033X_1X_3 - 0.0225X_2X_3.$$

$$Y_2 = -332.9069 + 3.5406X_1 + 65.9813X_2 + 2.8957X_3 - 0.0342X_1^2 - 4.6202X_2^2 - 0.0402X_3^2 - 0.1113X_1X_2 - 0.0104X_1X_3 + 0.0238X_2X_3.$$

The lack of fit test measures the failure of the model to represent data in the experimental domain at points which are not included in the regression (Rustrom *et al.*, 1991). None of the models exhibited lack of fit. The coefficient of determination (R^2) is the proportion of variability in the data explained or accounted for by the model (Montgomery, 1984). The model for the DH of shrimp proteins ($R^2 = 0.971$) indicated that only 2.9% of the total variation was not explained by the model. The R^2 value for nitrogen recovery (NR) was 0.951, indicating that only 4.9% of the total variation was not explained by the model. These models were considered adequate with satisfactory R^2 (>0.85) and significant F values. The coefficient of variation (CV) is the ratio of the standard error of estimate to the mean value of observed response expressed as a percentage. It is a measure of reproducibility of the models. As a general rule, a model can be considered reasonably reproducible if its CV is not

greater than 10% (Joglekar and May, 1987), as was the case in this study.

The estimated regression coefficients for each dependent variable obtained for shrimp proteins by multiple linear regression is given in Table 4.8. For DH, regression coefficients showed that temperature (X_1) and pH (X_2) had linear effects, and the three variables considered in this study, had quadratic effects at a level of significance of $p<0.01$. Similarly, temperature (X_1) and pH (X_2) had linear effects on NR, however, only temperature (X_1) and enzyme/substrate concentration ratio (X_3) had significant quadratic effects ($p<0.01$ and 0.05, respectively). Interactions were observed between temperature and enzyme/substrate concentration ($p<0.05$), temperature and pH ($p<0.10$) and pH and enzyme/substrate concentration ratio ($p<0.10$) on DH for shrimp. For NR, interaction was observed between temperature and pH ($p<0.10$), but interactions between temperature and enzyme/substrate concentration ratio as well as pH and enzyme/substrate concentration ratio were not significant ($p>0.1$). The largest values for the estimated regression coefficients for pH were 34.12 and 73.69, for DH and NR, respectively, and indicated that pH was the most important linear variable influencing these responses during preparation of shrimp protein hydrolysates. Thus, the linear variable effect decreased in the order pH > temperature > enzyme/substrate concentration ratio. The positive values of the linear factors X_1, X_2, and X_3 implied that the DH and NR increased with increasing values of these variables during hydrolysis.

The adequacy of the model for DH and NR of lumpfish proteins was also

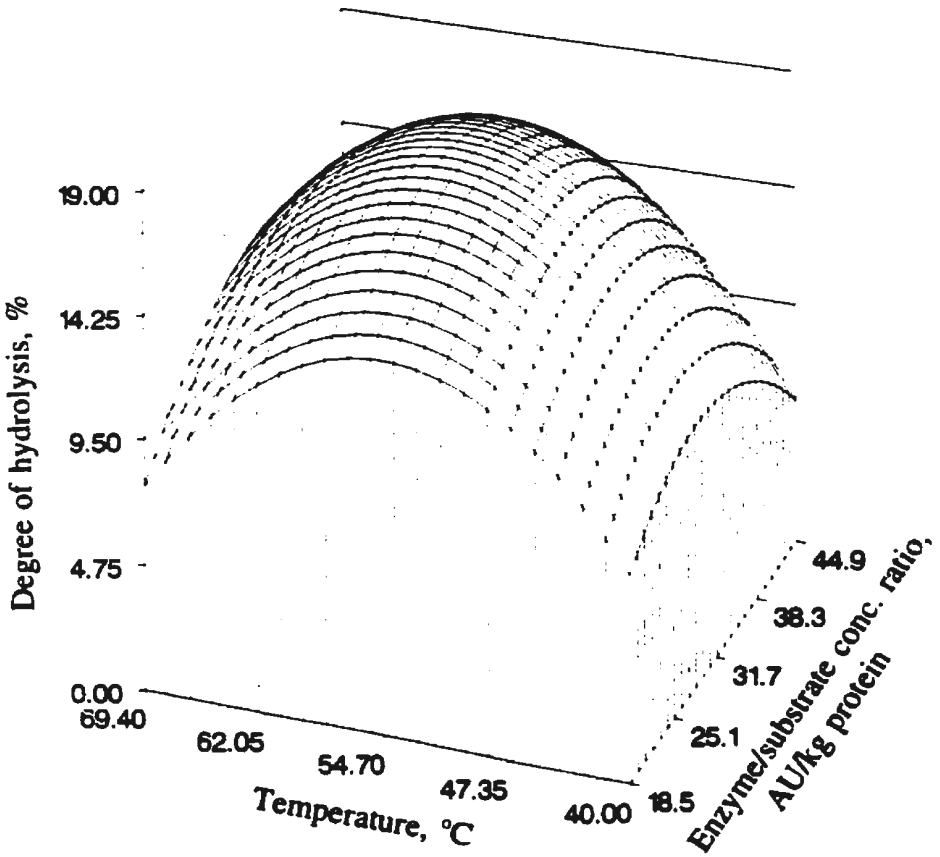
tested by examination of R^2 and lack of fit. The R^2 values were 0.954 and 0.924 for DH and NR, respectively, and indicated that only 4.6 and 7.6% of respective total variations for both responses were not explained by the model. The lack of fit was not significant ($P<0.05$), which also indicated that the models were adequate. The CV for DH and NR were 3.26 and 4.34%, respectively. This lends further support to the reproducibility of the models employed. The estimated regression coefficients for each independent variable obtained by multiple linear regression for lumpfish protein hydrolysate are given in Table 4.9. The regression coefficients showed that pH (x_2) and enzyme/substrate concentration ratio (x_3) had linear and quadratic effects ($p<0.01$) on DH of lumpfish proteins, respectively. However, only pH had significant ($p<0.05$) linear and quadratic effects on NR. Interaction between temperature and pH: and temperature and enzyme/substrate concentration ratio on the DH of lumpfish were not significant ($p>0.1$). Interaction was observed between temperature and enzyme/substrate concentration ratio ($p<0.05$) on NR for lumpfish, but interaction between temperature and pH as well as pH and enzyme/substrate concentration ratio were not significant ($p>0.1$). The largest values of estimated regression coefficient were obtained for pH. These values were 17.92 and 65.98 for DH and NR, respectively, therefore, indicates that pH was the most contributing variable which affected the above responses. The positive slopes for all variables indicated that DH and NR increased with increasing temperature, pH and enzyme/substrate concentration ratio.

4.2.4 3-D Response surface plotting and optimization based on canonical analysis

Three-dimensional response surface plots of DH and NR of shrimp proteins as a function of temperature, pH and enzyme/substrate concentration ratio are given in Figures 4.11 to 4.14. These figures showed that temperature, pH and enzyme/substrate concentration ratio shared the common trend of increasing the DH towards a maximum. A canonical analysis of the predicted quadratic polynomial models was performed in order to examine the overall shape of the response surface curves. The result was used to characterize the nature of the stationary points which indicated that both responses (DH and NR) for shrimp protein hydrolysate possessed maximum stationary points (Table 4.10). The maximum DH (17.8%) was predicted at a temperature of 57 °C, pH of 8.1 and enzyme/substrate concentration ratio of 33.1 AU/kg crude protein. The predicted maximum NR was 78.7% at 58 °C, pH 8.1 and enzyme/substrate concentration ratio of 31.9 AU/kg crude protein. These critical values of the three independent variables and the stationary points were located in the experimental region.

The relationship between the independent variables and responses for lumpfish protein hydrolysate is shown in the three-dimensional response surface representations generated for models developed for the responses (Figures 4.15 to 4.18). Canonical analysis of the lumpfish protein hydrolysate data indicated that all eigen values were negative. Thus, the stationary point also was maximum as those for the shrimp protein hydrolysate (Table 4.11). The maximum value of DH of 18.4% was predicted at 63 °C, pH of 8.1, and enzyme/substrate concentration ratio of 28.5 AU/kg crude protein.

Figure 4.11 Response surface and contour plots for the effect of temperature enzyme/substrate concentration ratio and pH on degree of hydrolysis of the prepared shrimp protein hydrolysate (pH = 8.0).


Degree of hydrolysis, %
19.00
14.25
9.50
4.75
0.00
69.40
62.05
54.70
47.35
40.00
Temperature, °C
18.5
25.1
31.7
38.3
44.9
Enzyme/substrate conc. ratio, AU/kg protein

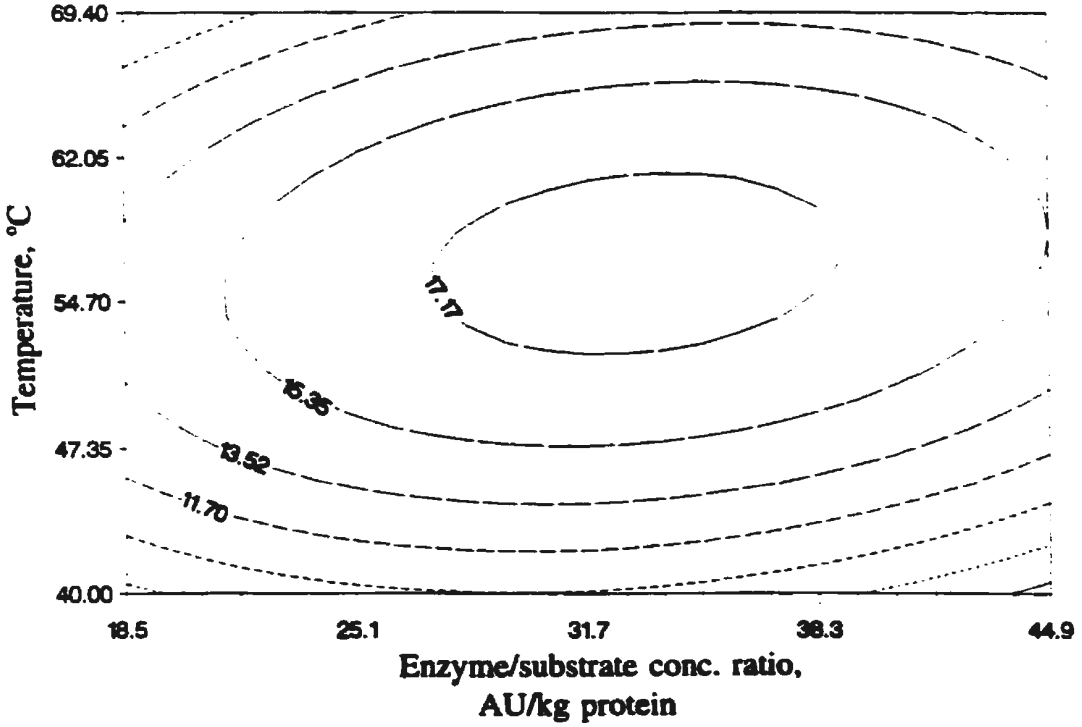
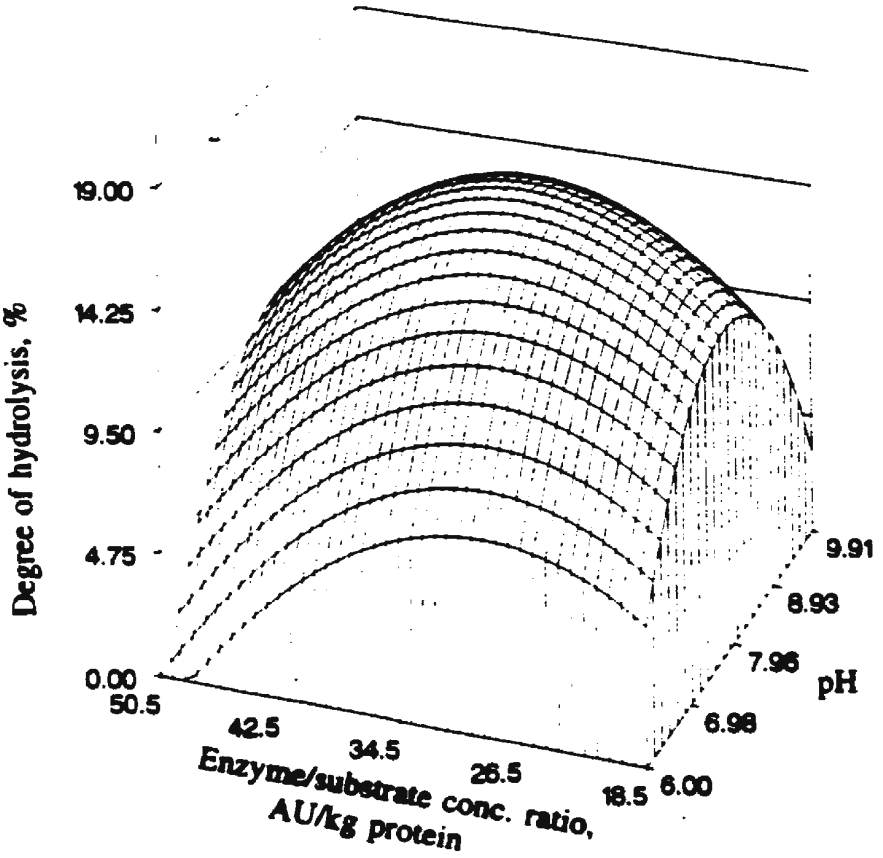

Temperature, °C
69.40
62.05
54.70
47.35
40.00
17.17
15.35
13.52
11.70
18.5
25.1
31.7
38.3
44.9
Enzyme/substrate conc. ratio,
AU/kg protein

Figure 4.12 Response surface and contour plots for the effect of enzyme/substrate concentration ratio, pH and temperature on degree of hydrolysis of the prepared shrimp protein hydrolysate (temperature = 57 °C).


Degree of hydrolysis, %
19.00
14.25
9.50
4.75
0.00
50.5
42.5
34.5
26.5
18.5
Enzyme/substrate conc. ratio, AU/kg protein
6.00
6.98
7.96
8.93
9.91
pH

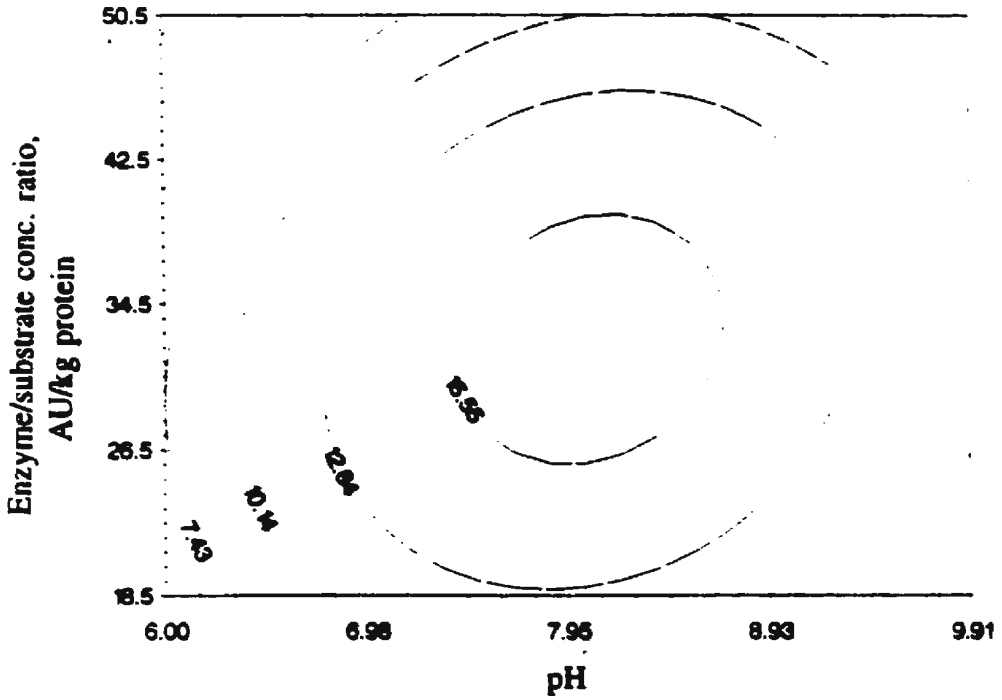
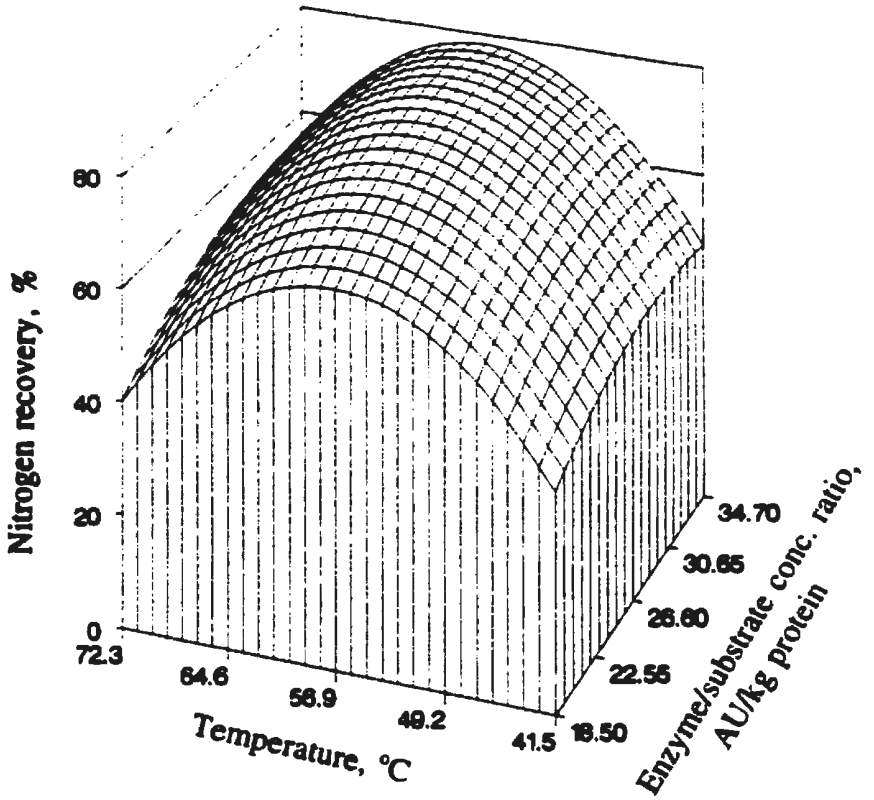

Enzyme/substrate conc. ratio, AU/kg protein
50.5
42.5
34.5
26.5
18.5
6.00
6.98
7.96
8.93
9.91
pH

Figure 4.13 Response surface and contour plots for the effect of temperature, enzyme/substrate concentration ratio and pH on nitrogen recovery of the prepared shrimp protein hydrolysate (pH = 8.1).


Nitrogen recovery, %
80
60
40
20
0
72.3
64.6
56.9
49.2
41.5
Temperature, °C
18.50
22.55
26.60
30.65
34.70
Enzyme/substrate conc. ratio,
AU/kg protein

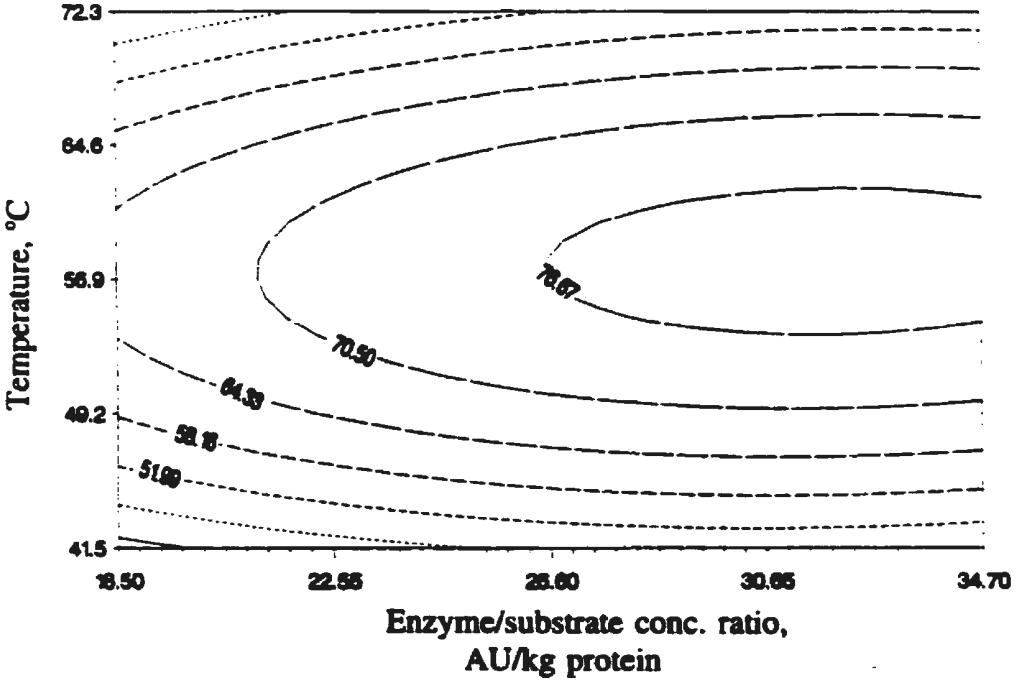
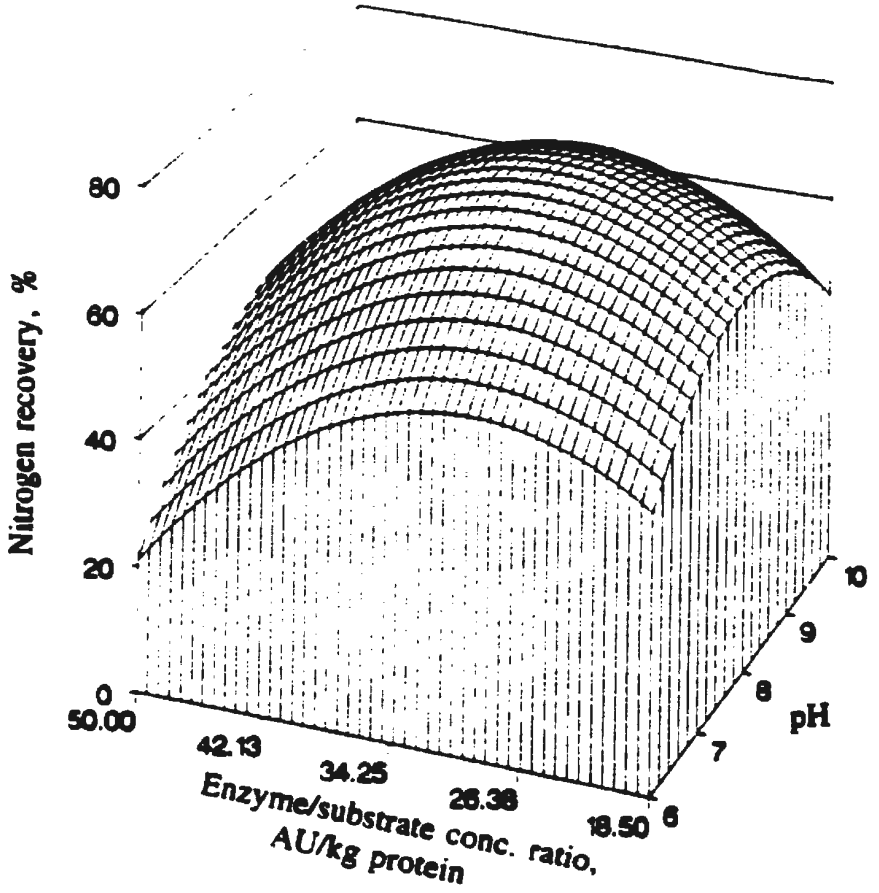

72.3
64.6
56.9
49.2
41.5
Temperature, °C
78.67
70.50
64.39
58.78
51.89
18.50
22.55
26.60
30.65
34.70
Enzyme/substrate conc. ratio,
AU/kg protein

Figure 4.14 Response surface and contour plots for the effect of enzyme/substrate concentration ratio, pH and temperature on nitrogen recovery of the prepared shrimp protein hydrolysate (temperature = 57 °C).


Nitrogen recovery, %
80
60
40
20
0
50.00
42.13
34.25
26.38
18.50
Enzyme/substrate conc. ratio,
AU/kg protein
6
7
8
9
10
pH

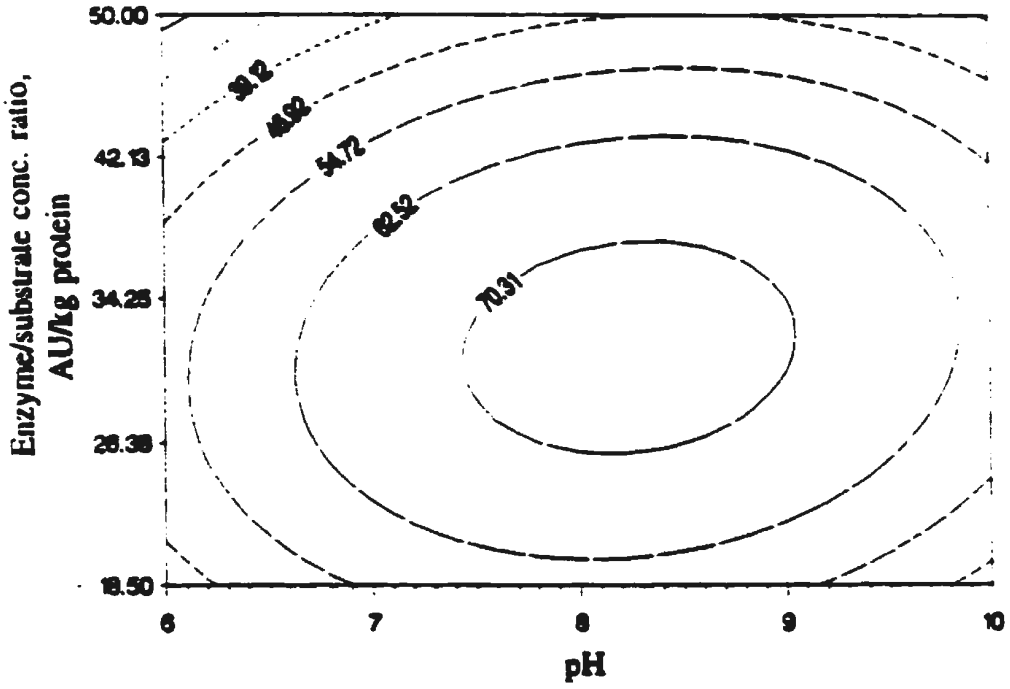
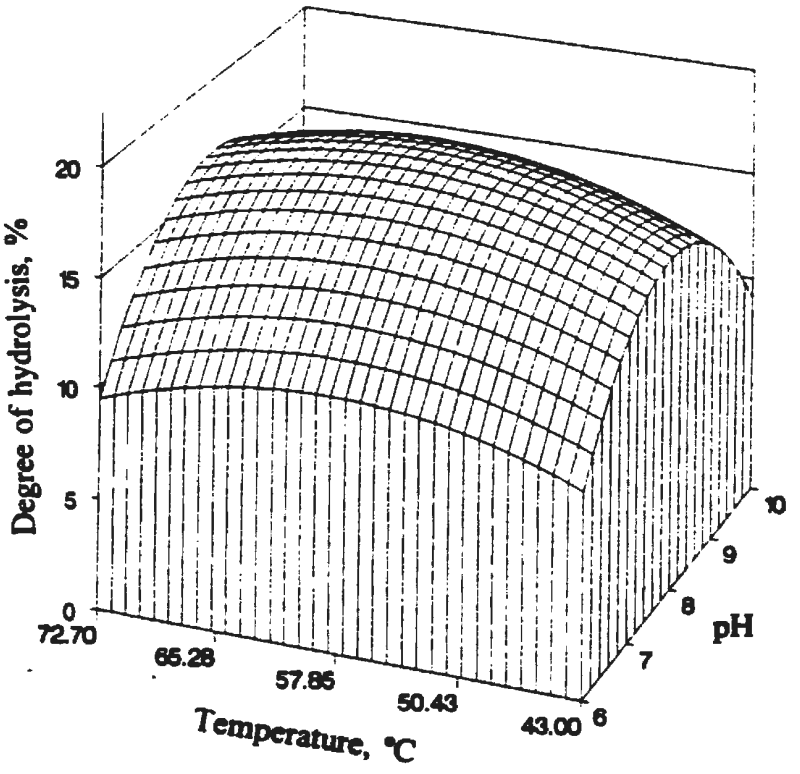

Enzyme/substrate conc. ratio,
AU/kg protein
50.00
42.13
34.25
26.38
18.50
30.12
46.92
54.72
62.52
70.31
6
7
8
9
10
pH

Figure 4.15 Response surface and contour plots for the effect of temperature, pH and enzyme/substrate concentration ratio on degree of hydrolysis of prepared lumpfish hydrolysate (enzyme/substrate concentration ratio = 28.5 AU/kg crude protein).


Degree of hydrolysis, %
20
15
10
5
0
72.70
65.28
57.85
50.43
43.00
Temperature, °C
6
7
8
9
10
pH

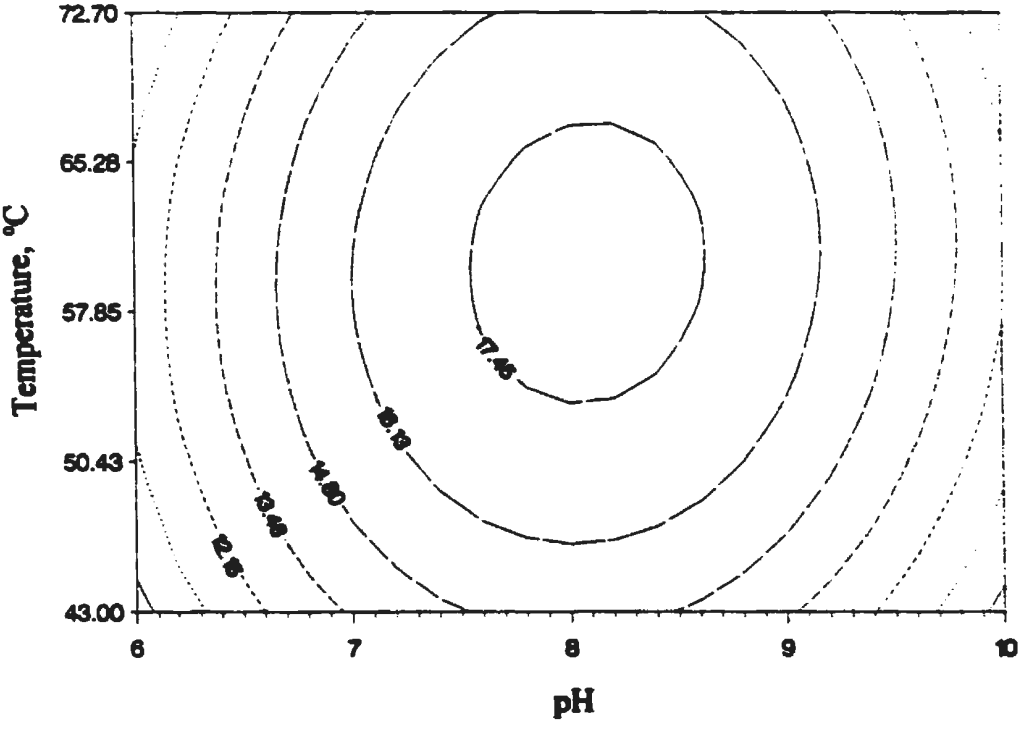
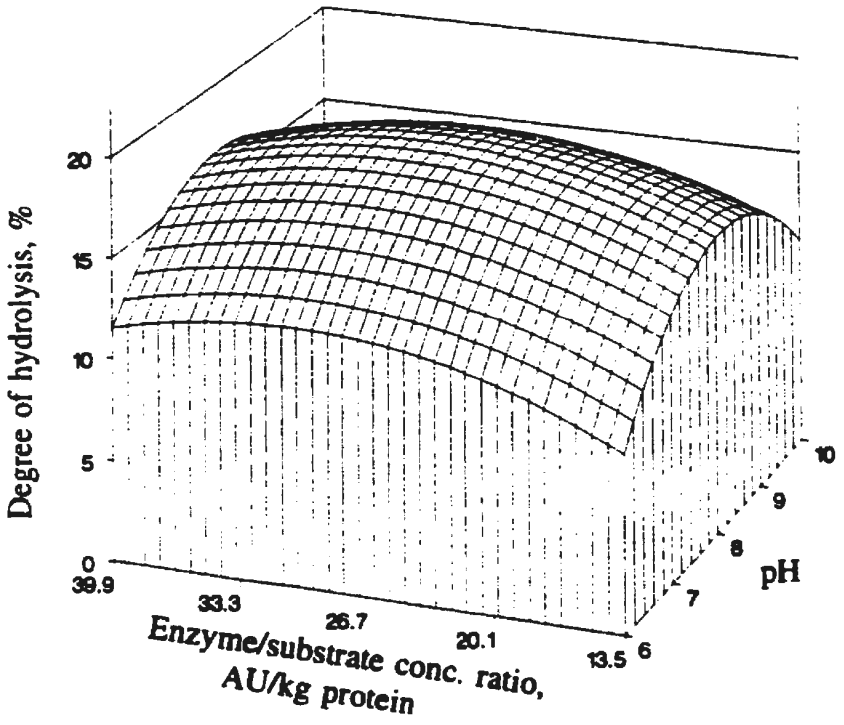

Temperature, °C
72.70
65.28
57.85
50.43
43.00
6
7
8
9
10
pH
17.45
16.10
14.80
13.48
12.15

Figure 4.16 Response surface and contour plots for the effect of enzyme/substrate concentration ratio, pH and temperature on degree of hydrolysis of prepared lumpfish protein hydrolysate (temperature = 63 °C).


Degree of hydrolysis, %
0
5
10
15
20
39.9
33.3
26.7
20.1
13.5
Enzyme/substrate conc. ratio,
AU/kg protein
6
7
8
9
10
pH

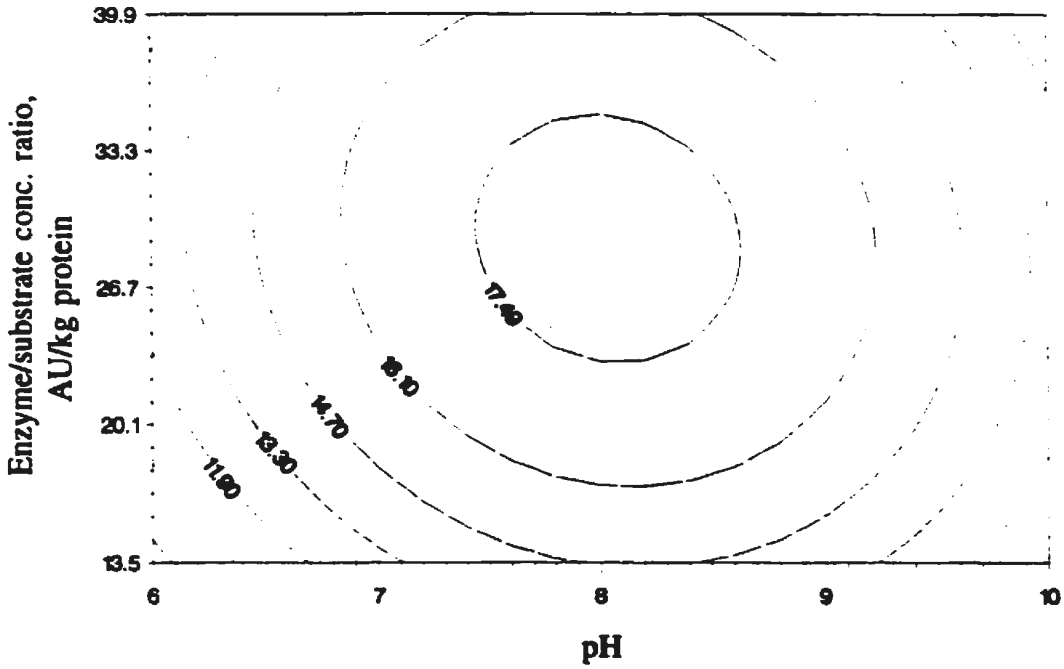
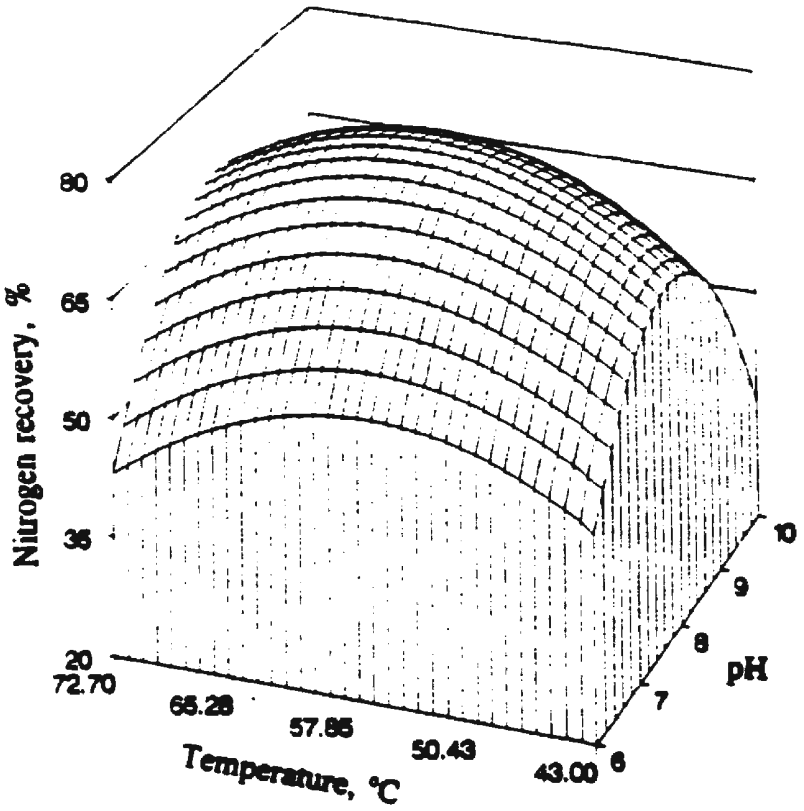

Enzyme/substrate conc. ratio,
AU/kg protein
39.9
33.3
26.7
20.1
13.5
17.40
16.10
14.70
13.30
11.90
6
7
8
9
10
pH

Figure 4.17 Response surface and contour plots for the effect of temperature, pH and enzyme/substrate concentration ratio on nitrogen recovery of the prepared lumpfish protein hydrolysate (enzyme/substrate concentration = 30.6 AU/kg crude protein).


Nitrogen recovery, %
80
65
50
35
20
72.70
65.28
57.86
50.43
43.00
Temperature, °C
6
7
8
9
10
pH

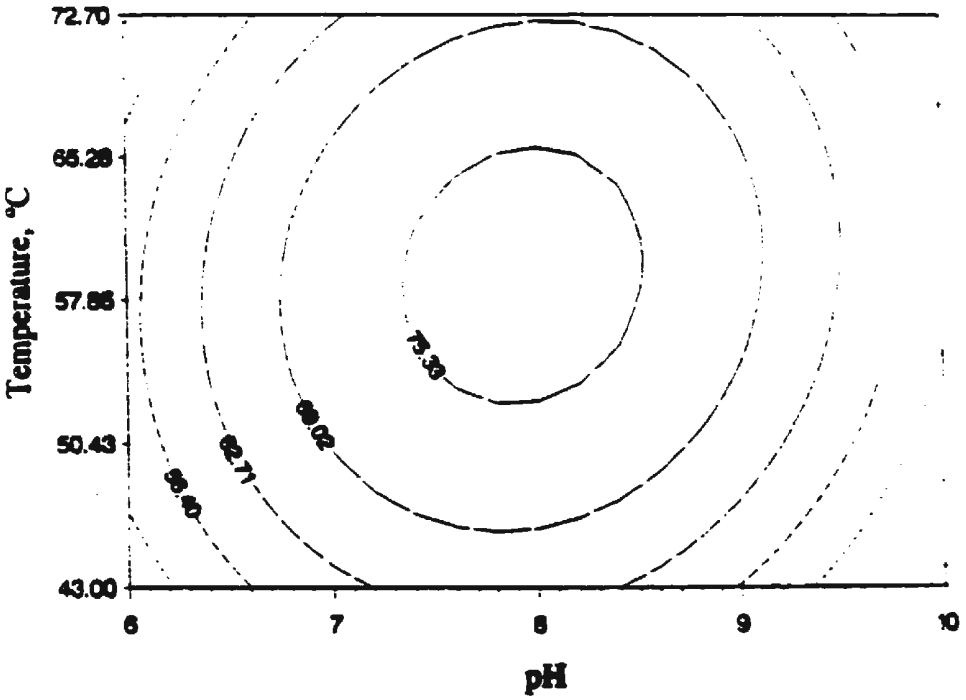
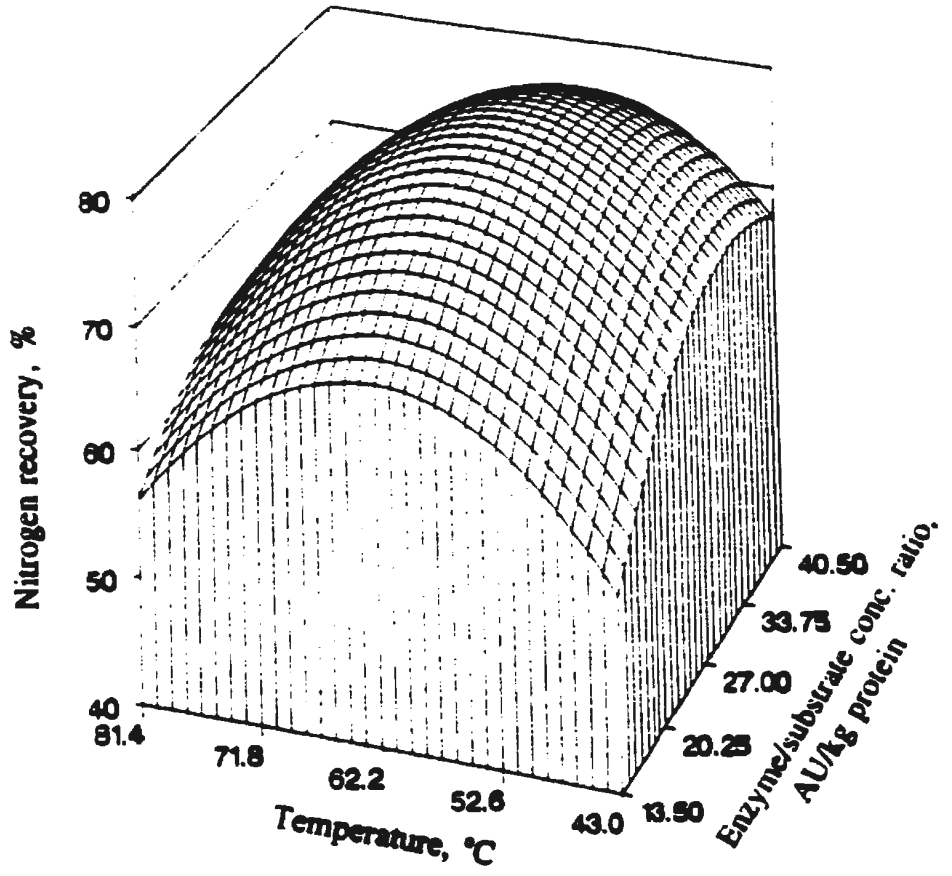
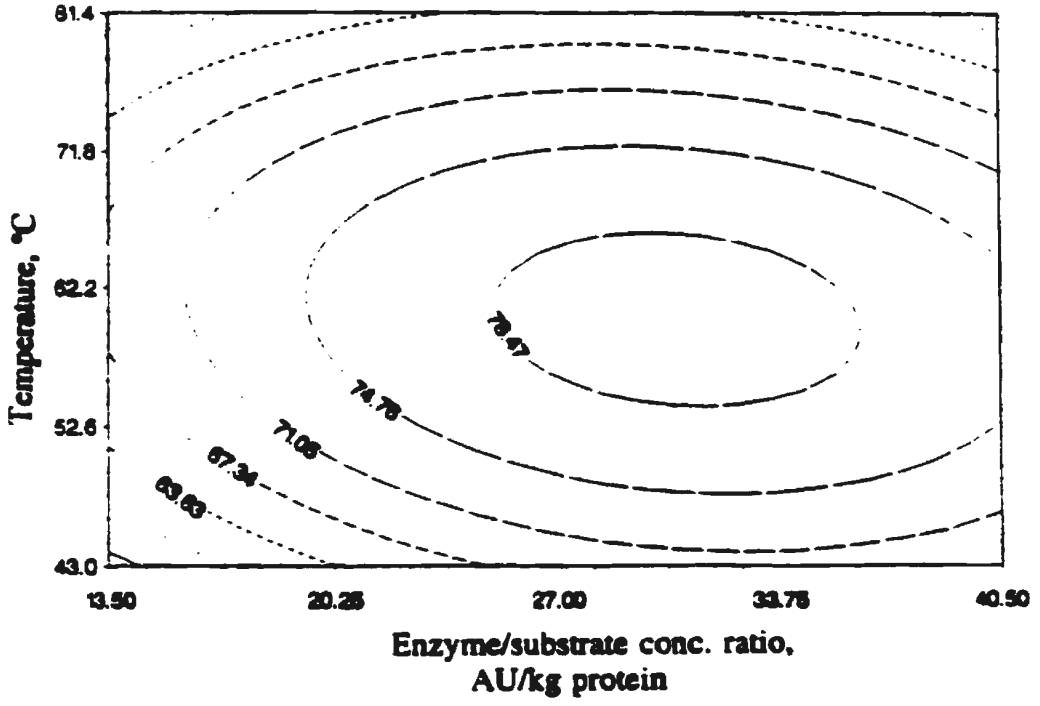

Temperature, °C
72.70
65.28
57.86
50.43
43.00
6
7
8
9
10
pH
73.39
69.02
62.71
56.40

Figure 4.18 Response surface and contour plots for the effect of temperature, enzyme/substrate concentration ratio and pH on nitrogen recovery of prepared lumpfish protein hydrolysate (pH = 8.0)


Nitrogen recovery, %
80
70
60
50
40
81.4
71.8
62.2
52.6
43.0
Temperature, °C
13.50
20.25
27.00
33.75
40.50
Enzyme/substrate conc. ratio,
AU/kg protein


Temperature, °C
81.4
71.8
62.2
52.6
43.0
78.47
74.76
71.05
67.34
63.63
13.50
20.25
27.00
33.75
40.50
Enzyme/substrate conc. ratio,
AU/kg protein

The predicted maximum NR was 78.7% at a temperature of 60 °C, pH of 7.9, and enzyme/substrate concentration ratio of 30.6 AU/kg crude protein. The contour plot of the data showed that these maxima were located in the experimental region. The contour plots derived from results of canonical analysis showed ellipsoidal contours at the maximum regions.

Verification experiments performed at the predicted optimum conditions showed reasonably close values for both responses of shrimp and lumpfish hydrolysates (Tables 4.10 and 4.11, respectively). The observed values of DH and NR for shrimp hydrolysate were 4.89 and 8.29%, respectively, of the predicted values. The observed values for lumpfish hydrolysate were 2.55 and 1.47% of the predicted values for DH and NR, respectively. These results revealed that the predicted values are reproducible and further confirms the adequacy of the predicted models.

4.3 Hunter colour parameters of hydrolysates

The colour of whole lumpfish, the starting material for production of lumpfish protein hydrolysate, is dark, with an Hunter L value of 30.2, when ground, as compared with 84.1 for the hydrolysate obtained from it (Table 4.12). The hydrolysate, once dehydrated, decolorized and deodorized, perhaps due to the removal of hemoproteins and other coloured matters, had a crystalline and clear appearance. This makes the product easily acceptable for use in food formulations where appearance might play an important role in acceptability of products. The Hunter a

Table 4.10 Predicted and observed values for response variables (degree of hydrolysis and nitrogen recovery) in hydrolysis of shrimp.

Response variables	Critical values of independent variables			Stationary point	Predicted point	Observed value[2]
	Temperature, °C	pH	Enz/substrate conc.[1]			
Degree of hydrolysis, %	57.6	8.1	33.1	maximum	17.78	18.65±1.04
Nitrogen recovery, %	57.9	8.1	31.9	maximum	78.74	72.21±2.17

[1] Enzyme/substrate concentration ratio, AU/kg crude protein.
[2] Mean ± SD (n=3).

Table 4.11 Predicted and observed values for response variables (degree of hydrolysis and nitrogen recovery) in hydrolysis of lumpfish.

Response variables	Critical values of independent variables			Stationary point	Predicted point	Observed value[2]
	Temperature, °C	pH	Enz/substrate conc.[1]			
Degree of hydrolysis, %	63.2	8.1	28.5	maximum	18.42	17.95±1.02
Nitrogen recovery, %	60.0	7.9	30.6	maximum	78.74	79.90±2.02

[1] Enzyme/substrate concentration ratio, AU/kg crude protein.
[2] Mean ± SD (n=3).

Table 4.12 Hunter **L. a. b** colour values of lumpfish and its protein hydrolysate[1].

	Hunter values		
Material	**L**	**a**	**b**
Ground lumpfish	30.2±0.4	1.5±0.1	6.2±0.1
Lumpfish hydrolysate	84.1±0.2	-1.3±0.1	10.1±0.1

[1]Results are mean values of 4 determinations ± standard deviation. Values in the same column are significantly ($P < 0.05$) different from one another.

and **b** values (for redness and yellowness, respectively) were similarly lowered in the washed meats, but to a lesser extent. In addition to the appearance, the undesirable fishy odour was virtually absent in the hydrolysate. However, hydrolysates prepared from shrimp retained a slight, but distinct shrimp odour, even after treatment with charcoal.

4.4 Composition of protein hydrolysates

4.4.1 Proximate composition of protein hydrolysates

The composition of ground shrimp and lumpfish and their protein hydrolysates is given in Table 4.13. The starting materials of shrimp and lumpfish, had 14.6 and 5.63% crude protein, respectively, but their resultant protein hydrolysates had a protein enrichment of 5 and 14-fold, respectively. The elevated protein content of the hydrolysates is due to removal of water and lipid from the starting material, thus concentrating the hydrolysed proteins. The lipid contents were lowered by 89.9 and 93.6%, in shrimp and lumpfish protein hydrolysates, respectively. Removal of a large portion of lipids would enhance the shelf life of the products as lipid oxidation and off-flavour development may be retarded by both the reduction in lipid content and generation of possibly antioxidative compounds; change in water activity might also influence lipid oxidation (Nawar, 1996). Low lipid content of protein hydrolysates from plant and animal sources have been reported and is one of the merits of protein hydrolysis (Mackie, 1982; Adler-Nissen, 1986a). However, the ash content was increased in the protein hydrolysates due to elevated sodium chloride which was

Table 4.13 Proximate composition of ground shrimp heads and lumpfish and their protein hydrolysates[1].

Species/Component	Starting material (%)	Protein hydrolysate[2] (%)
Shrimp		
Moisture	69.18±0.24	3.85±0.04
Crude Protein	14.64±0.16	74.32±1.64
Lipid	8.81±0.02	0.89±0.01
Ash	5.41±0.13	19.96±0.34
Lumpfish		
Moisture	90.55±0.38	4.55±0.04
Crude Protein	5.63±0.18	76.52±1.83
Lipid	2.51±0.05	0.16±0.02
Ash	1.20±0.04	18.33±0.32

[1]Results are mean values of 3 determinations ± standard deviation. Values in the same row are significantly ($P<0.05$) different from one another.

[2]Hydrolysis conditions for shrimp were temperature, 60 °C; pH, 8.1 and enzyme/substrate concentration ratio, 30 AU/kg crude protein; and those of lumpfish were temperature, 60 °C; pH, 8.0 and enzyme/substrate concentration ratio, 29 AU/kg crude protein.

formed primarily due to the addition of sodium hydroxide during the hydrolysis process followed by subsequent neutralization with hydrochloric acid. Increased ash content which is an indication of increased mineral level has been reported in other controlled enzyme-hydrolysed food proteins when NaOH was used (Mutilangi *et al.*, 1995; Shahidi *et al.*, 1995; Diniz and Martin, 1997).

4.4.2 Free amino acid content of protein hydrolysates

The content of free amino acids in shrimp heads and their protein hydrolysates is given in Table 4.14. The free amino acids were found to increase significantly ($p > 0.05$) in the hydrolysates as compared with the starting material. Over a 10-fold increase was noted for each of the amino acids analyzed. The hydrolysates of shrimp head proteins resulted in the release of free amino acids and short chain peptides. The degree of hydrolysis on free amino acid content of hydrolysates was expected to increase as the degree of hydrolysis increased. However, in hydrolysate 1 with DH = 12.7% and hydrolysate 2 with DH = 17.2% only a marginal effect on the content of free amino acids was observed (Table 4.14). This may be due to extensive hydrolysis which might have occurred in both products. The gel electrophoretic analysis indicated that most of the high molecular weight proteins were hydrolysed and reduced to very low-molecular-weight peptides and free amino acids after 5 min of hydrolysis. Thus, at DH = 17.2, shorter chain peptides were presumably formed from their longer chain counterparts with further production of only small amounts of free amino acids.

Table 4.14 The content of free amino acids of shrimp heads, and its protein hydrolysates (mg/100g sample)[1].

Amino acid	Shrimp heads	Hydrolysate 1	Hydrolysate 2
Alanine	42.14±0.6	267.1±6.95	285.6±3.95
Arginine	95.12±0.3	782.8±23.9	825.6±9.05
Aspartic acid	8.39±0.26	56.72±3.81	61.59±0.64
Asparagine	3.54±0.23	31.54±0.65	38.32±0.59
Cysteine	2.04±0.15	60.41±0.58	65.51±0.40
Glutamic acid	19.93±0.49	170.9±0.85	199.3±2.55
Glutamine	14.83±0.4	80.75±0.89	97.42±0.81
Glycine	114.2±2.13	785.8±7.75	825.6±9.05
Histidine	7.36±0.12	92.67±6.17	97.22±0.82
Hydroxyproline	0.67±0.0	3.67±0.12	4.05±0.11
Isoleucine	15.61±0.49	113.2±1.90	123.3±0.85
Leucine	21.22±0.57	296.0±5.10	324.5±2.25
Lysine	11.62±0.07	172.7±1.85	197.8±1.51
Methionine	9.63±0.33	80.12±0.73	126.9±2.65
Phenylalanine	11.9±0.28	216.5±1.31	230.3±2.05
Proline	86.75±0.86	736.2±21.1	771.0±2.60
Serine	9.92±0.27	99.9±0.65	104.7±0.53
Taurine	75.25±0.52	555.3±18.7	620.7±6.20
Threonine	7.05±0.18	101.1±0.45	109.8±0.45
Tryptophan	6.84±0.38	79.9±1.65	104.4±3.67
Tyrosine	12.64±0.26	135.5±0.75	149.9±0.81
Valine	17.81±0.21	134.1±2.45	152.3±1.95
Total	**594.4**	**5053**	**5515**

[1]Results are mean values of 3 replicates ± standard deviation. Values in each row are significantly ($p<0.05$) different from one another.

The lumpfish hydrolysate also had over a 10-fold increase in its free amino acid content as compared with its unhydrolysed counterpart (Figure 4.15). Free amino acids are flavour enhancers, and also participate in reactions involving formation of volatile flavour compounds in thermally processed foods (Erickson, 1992). Almost all free amino acids elicit taste which varies from bitter to sweet, depending on the individual amino acid tested. Glycine and alanine elicit a strong sweet taste (Kato *et al.*, 1989). It is thought that the taste of these amino acids is due to their ability to bind to the sweet substance receptors of the tongue. Glutamic acid which is usually present in large quantities in hydrolysates does not have a substantial taste itself, but its salt dissociates in water and elicits the umami flavour (Weir, 1986; Kato *et al.*, 1989). Perhaps the most important aspect of amino acid composition in flavour development, aside from their direct taste effect, is due to their reaction with carbonyl compounds in Maillard reactions.

The degree of hydrolysis has a direct influence on the amount of free amino acids released during hydrolysis. The type of enzyme used would also have an important bearing on the amount of free amino acids produced. Enzymes with narrow specificity such as Neutrase or some exopeptidases may produce low amounts of free amino acids. However, the use of endopeptidase with broad specificity or enzymes having both endo- and exopeptidase characteristics would extensively hydrolyse proteins and produce large amounts of free amino acids (Adler-Nissen, 1986b).

Table 4.15 The content of free amino acids of ground lumpfish and its protein hydrolysate (mg/100 g sample)[1]

Amino acid	Ground lumpfish	Protein hydrolysate
Alanine	6.68±0.20	290±8.0
Arginine	2.96±0.04	166±4.7
Aspartic acid	4.03±0.06	75.4±6.0
Cystine	2.03±0.01	89.4±1.0
Glutamic acid	5.59±0.28	160±0.4
Glutamine	2.00±0.11	113±2.1
Glycine	3.45±0.08	136±7.3
Histidine	1.12±0.01	62.6±0.5
Hydroxyproline	1.09±0.02	95.7±5.1
Isoleucine	2.33±0.13	146±4.5
Leucine	4.04±0.07	279±6.2
Lysine	7.41±0.07	197±5.4
Methionine	1.75±0.09	171±4.0
Phenylalanine	2.18±0.05	178±6.2
Proline	2.38±0.17	26.6±0.0
Serine	4.45±0.16	119±3.1
Taurine	4.10±0.08	708±14.0
Threonine	4.51±0.07	123±2.6
Tryptophan	0.20±0.00	12.6±0.1
Tyrosine	2.09±0.05	131±3.8
Valine	3.42±0.08	128±1.8
Total	**67.8**	**3408**

[1] Results are mean values of 3 replicates ± standard deviation. Values in each row for each amino acid are significantly ($p<0.05$) different from one another.

4.4.3 Total amino acid content and protein nutritional value of protein hydrolysates

The amino acid profile of shrimp protein hydrolysate and its starting material is given in Figure 4.19. The amino acid composition of the hydrolysate was essentially similar to that of shrimp heads proteins used for the hydrolysate preparation, and no destruction of amino acids was noted, indicating that the protein quality of the starting material was not compromised due to hydrolysis. Similar profiles of amino acid composition of sunflower (Parrado *et al.*, 1993) and capelin (Shahidi *et al.*, 1995) hydrolysates have been reported, thus supporting the non-destructive effect of enzyme-assisted hydrolysis on amino acids. Controlled enzymatic hydrolysis by endo- and exopeptidases is more gentle than acid hydrolysis, the former has the advantage of preserving the qualities of native protein, without altering the amino acid profile (Boza *et al.*, 1995) The amino acid composition of lumpfish protein hydrolysate also indicates insignificant changes in the amounts of its individual amino acids as compared with those of the starting material (Figure 4.20). Furthermore, this type of hydrolysis, where adequate control of the factors involved in the process is possible, can render a protein hydrolysate rich in certain desired characteristics which are associated with the size of peptides.

The nutritional quality of protein hydrolysates and their starting materials with respect to computed protein efficiency ratio (PER) and predicted biological value (BV) is given in Table 4.16. The calculated PER values of shrimp and lumpfish protein

Figure 4.19 Total amino acid composition of shrimp heads and their protein hydrolysate.

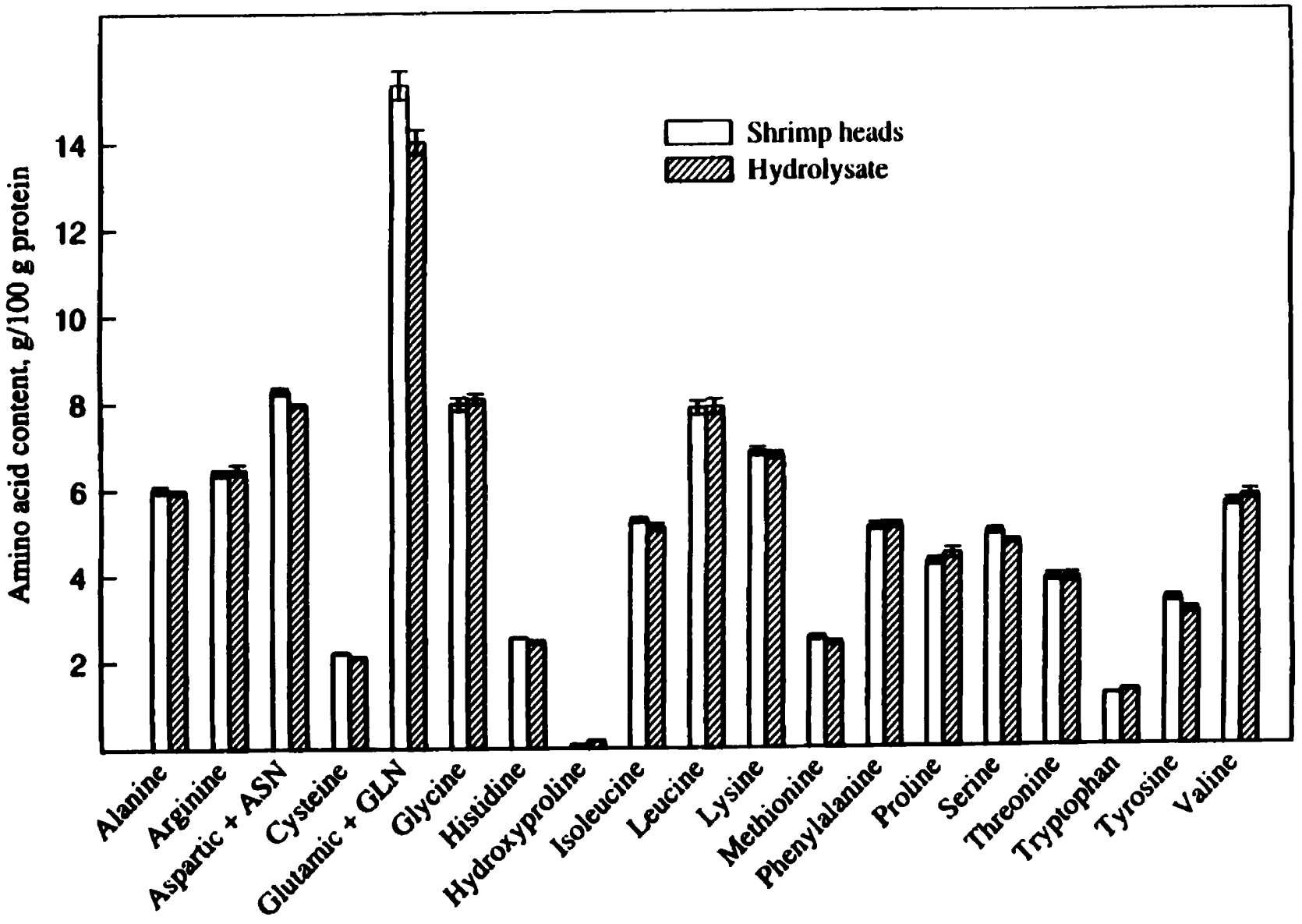

Amino acid content, g/100 g protein
14
12
10
8
6
4
2
Shrimp heads
Hydrolysate
Alanine
Arginine
Aspartic + ASN
Cysteine
Glutamic + GLN
Glycine
Histidine
Hydroxyproline
Isoleucine
Leucine
Lysine
Methionine
Phenylalanine
Proline
Serine
Threonine
Tryptophan
Tyrosine
Valine

Figure 4.20 Total amino acid composition of lumpfish and its protein hydrolysate

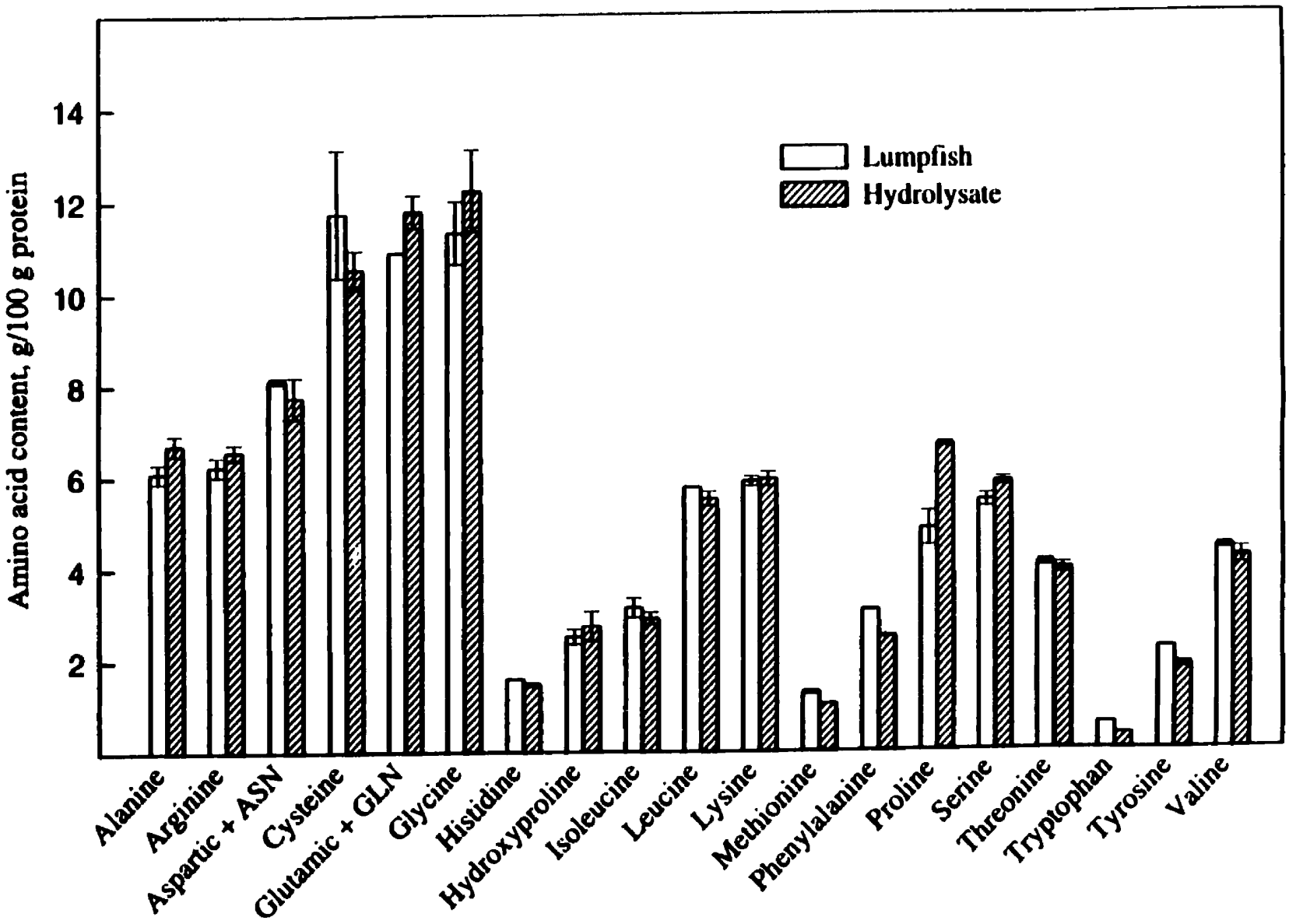

Amino acid content, g/100 g protein
14
12
10
8
6
4
2
Lumpfish
Hydrolysate
Alanine
Arginine
Aspartic + ASN
Cysteine
Glutamic + GLN
Glycine
Histidine
Hydroxyproline
Isoleucine
Leucine
Lysine
Methionine
Phenylalanine
Proline
Serine
Threonine
Tryptophan
Tyrosine
Valine

Table 4.16 Predicted protein nutritional values of shrimp and lumpfish hydrolysates in comparison with those of their starting materials.

Parameter	Shrimp heads proteins	Shrimp protein hydrolysate	Lumpfish proteins	Lumpfish protein hydrolysate
PER[1] Equation 1	2.83	2.82	2.10	1.99
Equation 2	2.83	2.82	2.10	2.01
BV[2]	82.6	94.8	30.9	26.1

[1]Protein efficiency ratio values were calculated using equations 1 and 2 given in section 3.8.1.
[2]Predicted biological values were calculated using equation proposed by Mørup and Olesen (1976) and given in section 3.8.1.

hydrolysates, using equations 1 and 2 (section 3.8.1) as proposed by Lee *et al.* (1978), show similar values when compared with those of their unhydrolysed starting proteins. However, a marginal decrease in PER values of 5.2 and 4.3%, based on equations 1 and 2, respectively, were noted for lumpfish protein hydrolysate as compared with their starting proteins. The nutritional value of the protein was further assessed using the amino acid score (Table 4.17) and biological value. An indication that the shrimp protein hydrolysate has high nutritional value is displayed by the amino acid requirement which meets the recommended reference values provided by FAO/WHO/UNU (1985). This was further confirmed by the amino acid score which was over 100% for all essential amino acids of shrimp protein hydrolysate (Table 4.17). The amino acid score and biological value further demonstrated that the protein quality of the shrimp protein hydrolysates was not compromised due to hydrolysis. However, lower than 100% amino acid scores were noted for histidine, leucine, phenylalanine + tyrosine and tryptophan in lumpfish protein hydrolysate. The amino acid score of tryptophan was 29%, thus making it the limiting amino acid in lumpfish protein hydrolysate.

4.5 Functional properties of protein hydrolysates

4.5.1 Solubility

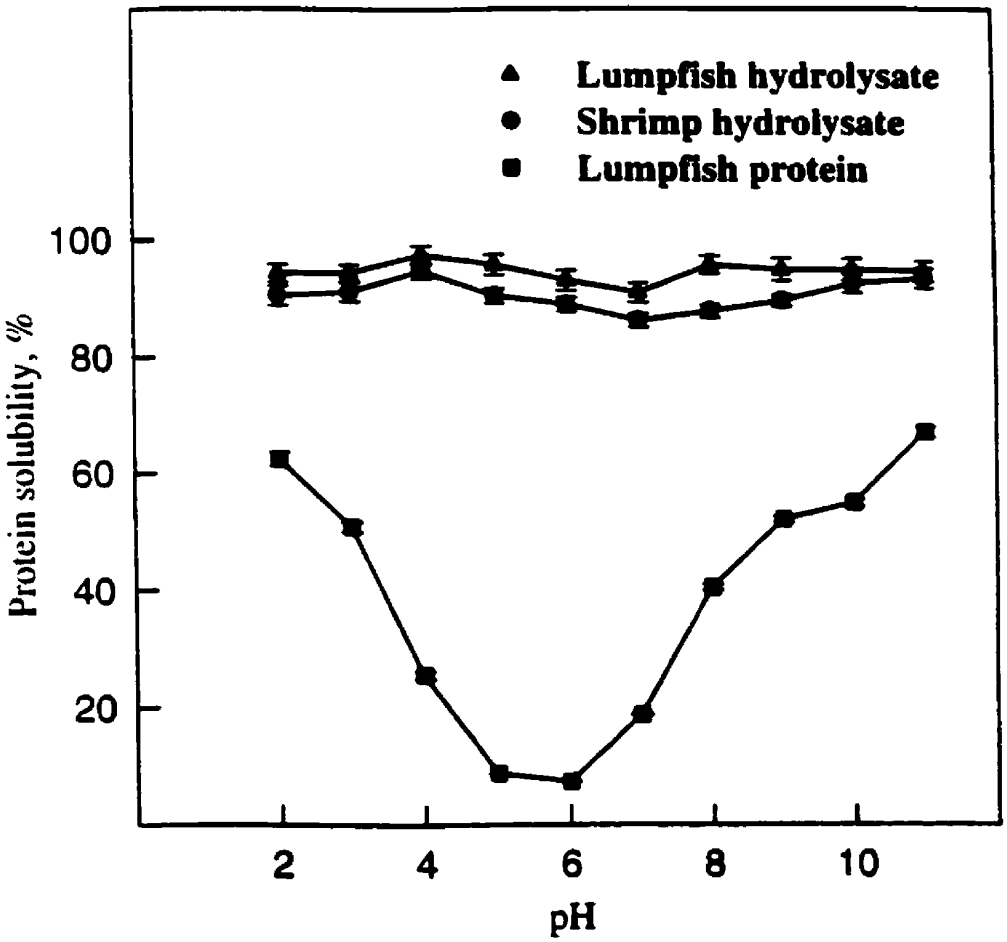
The solubility of protein hydrolysates in water was enhanced due to enzymatic hydrolysis. Figure 4.21 shows the solubility profile of shrimp protein hydrolysate as

Table 4.17 Amino acid scores of shrimp and lumpfish protein hydrolysates (%).

Amino acid	Reference[1]	Shrimp protein hydrolysate	Amino acid score[2]	Lumpfish protein hydrolysate	Amino acid score[2]
Histidine	1.9	2.44	128.4	1.47	7.37
Isoleucine	2.8	5.09	181.8	2.90	103.6
Leucine	6.6	7.88	119.4	5.48	83.03
Lysine	5.8	6.74	116.2	5.91	101.9
Methioneine + Cysteine	2.5	4.49	179.6	11.55	462.0
Phenylalanine + Tyrosine	6.3	8.22	130.5	4.30	68.25
Threonine	3.4	3.87	113.8	3.93	115.6
Tryptophan	1.1	1.26	114.5	0.32	29.09
Valine	3.5	5.78	165.1	4.18	119.4
Total	**33.9**	**45.77**		**40.04**	

[1]FAO/WHO/UNU (1985). [2]Amino acid score was calculated according to FAO/WHO (1991), see section 3.8.1.

Figure 4.21 Solubility profiles of shrimp and lumpfish hydrolysates at different pH values.


Lumpfish hydrolysate
Shrimp hydrolysate
Lumpfish protein
Protein solubility, %
100
80
60
40
20
2
4
6
8
10
pH

well as unhydrolysed lumpfish protein and its corresponding hydrolysates. The protein hydrolysates displayed high solubility in the pH range studied (2 to 11). Over 90% of both hydrolysates remained soluble in water in the pH range examined. Similarly, high solubility values for Alcalase-hydrolysed sardine (Quaglia and Orban, 1987b) and capelin (Shahidi *et al.*, 1995) have been reported. The solubility of lumpfish proteins was minimum at pH 5.0-6.0 and increased on both sides of this range. The low solubility of myofibrillar proteins at this pH is due to precipitation at their isoelectric points which are approximately the same. Minimum and maximum solubilities of lumpfish hydrolysates were 91 and 97.5% at pH 4 and 7, respectively. The enhanced solubility of protein hydrolysates may be due to high levels of free amino acids and peptides formed during the process as well as increase in ionizable groups during hydrolysis (Mutilangi *et al.*, 1996). The protein hydrolysates also showed improved thermostability as compared with their unhydrolysed counterparts (data not shown). There were obviously structural modification of the native proteins and interaction between hydrolysate components and water, as a result of the hydrolysis which prevented their coagulation and precipitation. The observed enhancement in thermostability may be a reflection of increased protein-water and peptide-water interactions.

4.5.2 Other functional properties

Selected functional properties of shrimp and lumpfish protein hydrolysates are presented in Table 4.18. The emulsifying properties as well as fat adsorption,

Table 4.18 Selected functional properties of lyophilized shrimp and lumpfish protein hydrolysates[1].

Functionality[2]		Percentage	
		Shrimp hydrolysate	Lumpfish hydrolysate
Fat adsorption		185±3.6	156±3.8
Moisture adsorption[3] 80% RH		6.1±0.1	3.3±0.0
Emulsifying capacity		59.8±0.5	59.7±0.8
Emulsion stability		96.2±0.6	89.3±0.4
Whippability		97.2±0.6	65.5±0.8
Foam stability	0.5 min	80±1.9	97.1±2.1
	5 min	48±0.4	76.5±0.3
	40 min	8±0.3	4±0.1
	60 min	8±0.2	0
	80 min	0	0

[1]Results are mean values of 3 replicates ± standard deviation.
[2]Slow addition of water to 100 g hydrolysate indicated that 35.3±0.3 and 31.7±0.4 mL water was needed to make a ball, 57.0±0.5 and 48.9±0.4 mL to make a paste and 72.0±0.6 and 61.0±0.6 mL to fully dissolve it.
[3]RH, relative humidity.

moisture adsorption, whippability and foam stability are functional properties which are influenced by solubility of protein hydrolysates in water. Fat adsorption was 185% for shrimp hydrolysate as compared with 156% for its lumpfish counterpart. The shrimp protein hydrolysate was also more hygroscopic, having 85% higher moisture adsorption as compared with lumpfish protein hydrolysate. However, the emulsifying capacity of both hydrolysates were same, thus indicating that they would serve as good emulsifiers. By virtue of their amphiphillic nature, some proteins are capable of orienting at the polar-nonpolar interface and are good emulsifiers (Mangino, 1994). The reduction of interfacial tension, which is essential for emulsification, is governed by three consecutive or concurrent processes: diffusion of protein molecules to and attachment at the interface; spreading or unfolding of the adsorbed molecules, and their molecular rearrangement at the surface (Tornberg, 1978). Protein hydrolysates seem to possess the desired characteristics required for these processes to occur.

Shrimp and lumpfish hydrolysates were also effective at 96 and 98%, respectively, in stabilizing the emulsions formed from corn oil and protein hydrolysate. Emulsion stabilizing effect exerted by a protein is governed by different molecular properties of such proteins, with regards to hydrophobicity, flexibility and amino acid composition. In case of milk proteins, both emulsifying activity and emulsion stability increased with increasing ionic strength (Klemaszewski *et al.*, 1992). The surface activity of the peptides results from a balance of the hydrophobic

and hydrophillic amino acid residues constituting the molecule (Panyam and Kilara, 1996). Different proteins and hydrolysates have different amino acid contents, and hence their surface activity would vary and the resultant variation in emulsion stability of shrimp and lumpfish hydrolysates may be attributed to the existing differences in their amino acid composition.

The capacity of hydrolysates to form foams is expressed as whippability; this was better performed by shrimp hydrolysate than its lumpfish counterpart. However, lumpfish hydrolysate was more effective in stabilizing the foam formed during the first 5 min of quiescent period. While after 0.5 min, 97% of the foam formed with lumpfish hydrolysate was stable, only 80% of that formed with shrimp hydrolysate was stable during the same period. The molecular properties which are relevant for foaming are similar to those required for emulsification. The factors deemed necessary for foaming include solubility, segmental flexibility to facilitate unfolding at the interface, disposition of charged and polar groups to prevent the close approach of bubbles which allow hydration and also steric effects (German and Phillips, 1994). The factors are present to a certain extent in the hydrolysates, hence their marginal foaming characteristics are noted. Limited proteolysis yields peptides with improved foaming properties. The foaming ability of whey protein improved with limited hydrolysis, but the stability of the foam decreased (Kuehler and Stine, 1974).

Protein supplements in food formulations are expected to perform a functional role as well as a nutritional one. With the use of appropriate enzymes and control of

hydrolysis conditions, the functionality of protein hydrolysates can be tailored to suit the specific needs of particular food formulations. Protein hydrolysates are finding new commercial applications in a number of formulated foods (Blendford, 1994), and the unique functional properties of protein hydrolysate will further enhance their use in a variety of products.

4.5.3 Influence of protein hydrolysates and phosphates on cooking yield and drip volume of mechanically deboned chicken meat (MDCM)

The cooking yield of MDCM was influenced by the presence of protein hydrolysates. The influence of the commonly used phosphates in the food industry, namely trisodium polyphosphate (TSPP), sodium tripolyphosphate (STPP) and sodium hexametaphosphate (SHMP) on cooking yield of MDCM was compared with that of shrimp protein hydrolysate. As shown in Figure 4.22, TSPP and STPP were more efficient in improving the cooking yield than shrimp protein hydrolysate at the same concentration. However, since only 0.5% polyphosphate is permitted in meat products, shrimp protein hydrolysate may be used at higher concentration to exert a better effect in improving cooking yield than the phosphates at its permissible levels.

Lumpfish protein hydrolysate, however, also improved the cooking yield of MDCM, but to a lesser extent than shrimp hydrolysate. Shrimp protein hydrolysate improved the cooking yield from 75.8% in the absence of hydrolysate to 119% in the presence of 3.0% hydrolysate. Lumpfish hydrolysate (3%) increased the cooking yield from 73.8% to 86.6% (Figure 4.23).

Figure 4.22 Influence of tetrasodium pyrophosphate (TSPP), sodium tripolyphosphate (STPP), sodium hexametaphosphate (SHMP) or shrimp protein hydrolysate (SHPH) at different concentrations on cooking yield of mechanically deboned chicken meat (MDCM). Values having the same letter at each concentration level, are not significantly ($p>0.05$) different from one another.

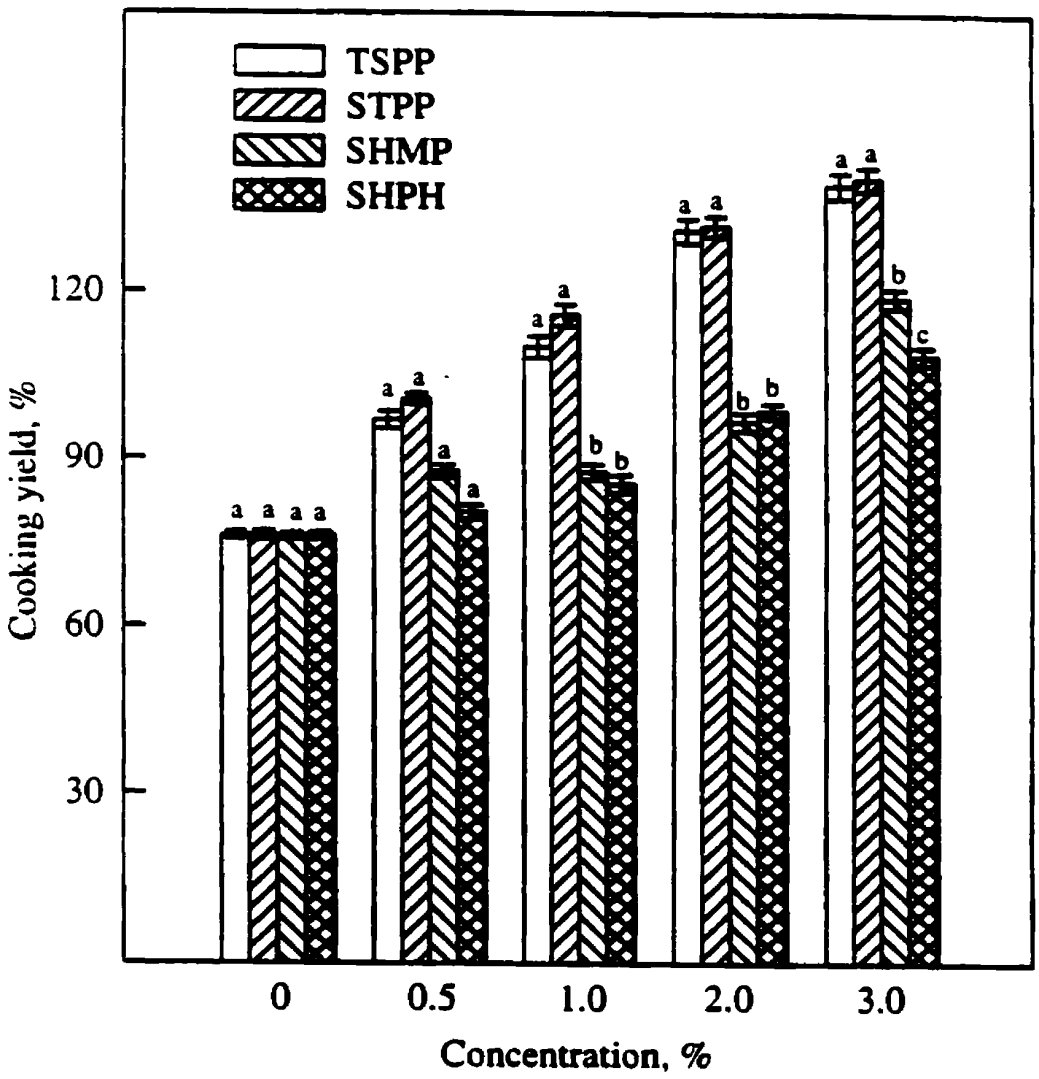
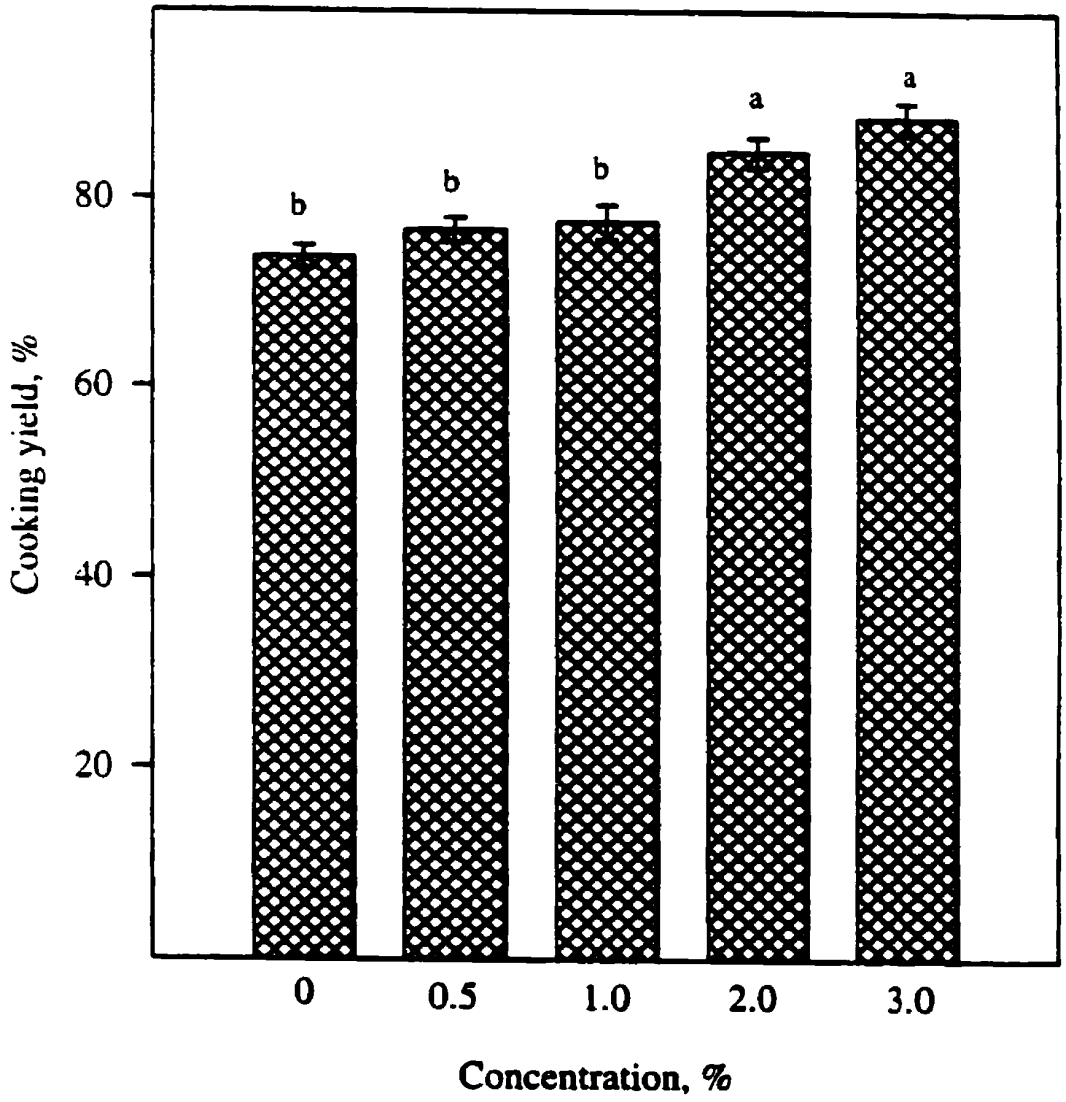

TSPP
STPP
SHMP
SHPH
Cooking yield, %
120
90
60
30
0
0.5
1.0
2.0
3.0
Concentration, %

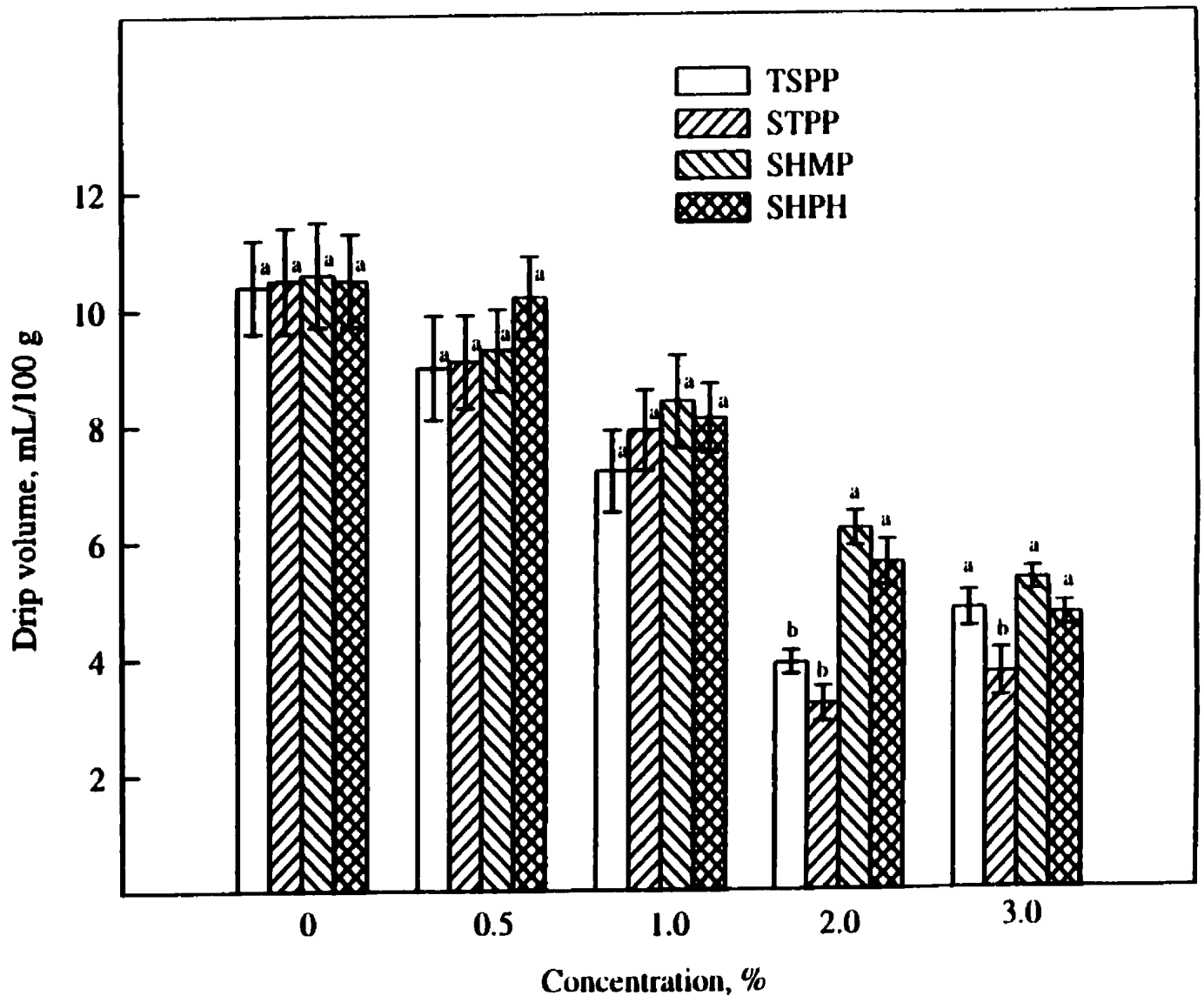
Figure 4.23 Influence of lumpfish hydrolysate at different concentrations on cooking yield of mechanically deboned chicken meat (MDCM). Values having the same letter are not significantly ($p>0.05$) different from one another.


Cooking yield, %
80
60
40
20
b
b
b
a
a
0
0.5
1.0
2.0
3.0
Concentration, %

The dripping loss of meat systems after thermal processing for 45 min at 85 °C with 0-3 % (w/v) of TSPP, STPP, SHMP or shrimp protein hydrolysate are shown in Figure 4.24. The drip volume displayed a minimum for TSPP and STPP at 2.0% concentration, while SHMP and shrimp protein hydrolysate did not show such a minimum in the range of concentrations considered (0-3 %). The polyphosphates, TSPP, STPP and SHMP, decreased the drip volume by approximately 69.5, 61.8 and 55.2%, respectively, compared with that of MDCM, which was heat-processed in the absence of phosphates. However, shrimp protein hydrolysate, at 3%, decreased the drip volume of MDCM by 50.9%.

The polyphosphates, TSPP and STPP have been shown to be most effective in increasing water-binding capacity of beef rolls (Trout and Schmidt, 1984) and mechanically separated seal meat (Shahidi and Synowiecki, 1997). The findings in this study lend further support to those reported by these authors. In the presence of phosphates the cooking loss of MDCM was minimum at a phosphate concentration of 2-3%, which is much higher than the 0.5% level permitted by the USDA (1982). The drip volume of MDCM at 0.5% shrimp protein hydrolysate was comparable to those of the phosphates used in this study. Thus, shrimp protein hydrolysate may serve as an alternative to phosphates as an ingredient which reduces exudates during heat processing of muscle food products. The role of protein hydrolysates in this regard may be attributed to the ionizable amino and carboxyl groups brought about by the hydrolysis process, which increased the hydrophilicity of meats containing

Figure 4.24 The drip volumes of mechanically deboned chicken meat (MDCM) cooked with different amounts of tetrasodium pyrophosphate (TSPP), sodium tripolyphosphate (STPP), sodium hexametaphosphate (SHMP) or shrimp protein hydrolysate (SHPH). Values having the same letter at each concentration level, are not significantly ($p>0.05$) different from one another.

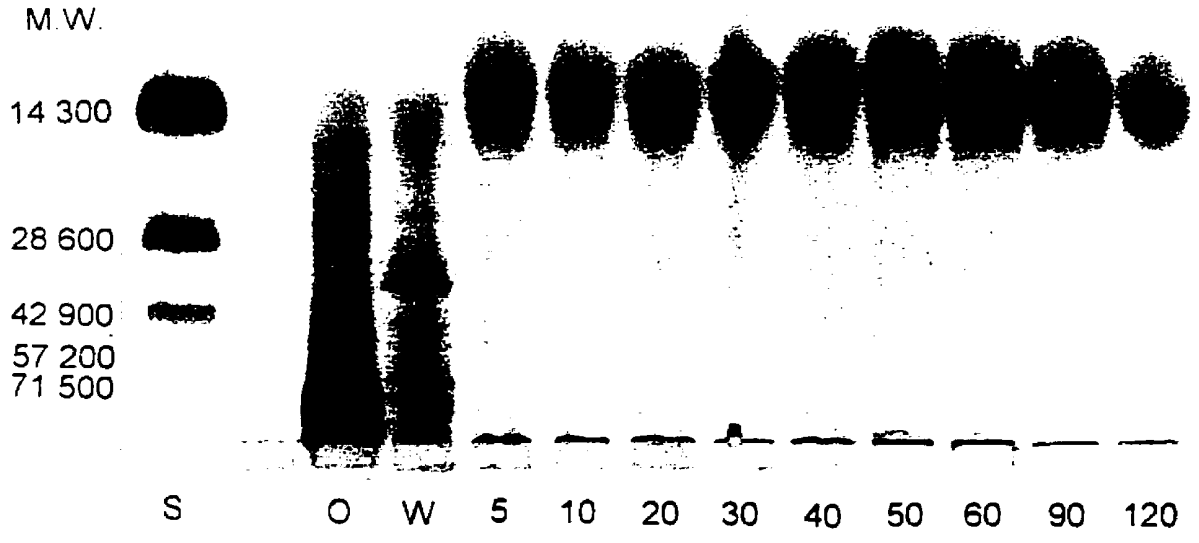

TSPP
STPP
SHMP
SHPH
Drip volume, mL/100 g
12
10
8
6
4
2
0
0.5
1.0
2.0
3.0
Concentration, %

hydrolysates (Phillips and Beuchat, 1981). Furthermore, possible interaction of divalent ions such as calcium and magnesium with hydrolyzed peptides and protection against formation of myosin-actin complex also prevents the loss of bound water from meat proteins (Schnepf, 1992).

4.6 Hydrolysis pattern on sodium dodecyl sulphate polyacrylamide gel (SDS-PAG) electrophoresis

The gel electrophoresis patterns of the hydrolysis of shrimp proteins is given in Figure 4.25. Alcalase caused a rapid hydrolysis of the native shrimp proteins as indicated by the disappearance of high-molecular-weight bands on the gels. After 5 min of hydrolysis, most of the proteins were hydrolysed to short chain peptides, of low molecular weight, a pattern which continued for up to 120 min. Benjakul and Morrissey (1997) reported a similar trend for Alcalase-assisted hydrolysis of Pacific whiting fish waste proteins. However, Neutrase which has a narrower specificity (Adler-Nissen, 1986a) showed lower hydrolytic activity when compared with Alcalase. Hence, the reduction in the high-molecular-weight proteins of Pacific whiting waste solids was less drastic and afforded bands ≤ 30 000 Da. The degree of hydrolysis of proteins does not seem to play a prominent role in the molecular weight distribution pattern of the hydrolysates. This may be explained by broad specificity of the Alcalase enzyme used in the hydrolysis (Adler-Nissen, 1986a). This observed pattern for Alcalase-assisted hydrolysis of shrimp was also found for lumpfish protein

Figure 4.25 Electrophorectrogram of shrimp protein hydrolysis pattern. S, authentic markers; O, original shrimp protein; W, water extracted shrimp proteins; and hydrolysates obtained at 5, 10, 20, 30, 40, 50, 60, 90 and 120 min, respectively.

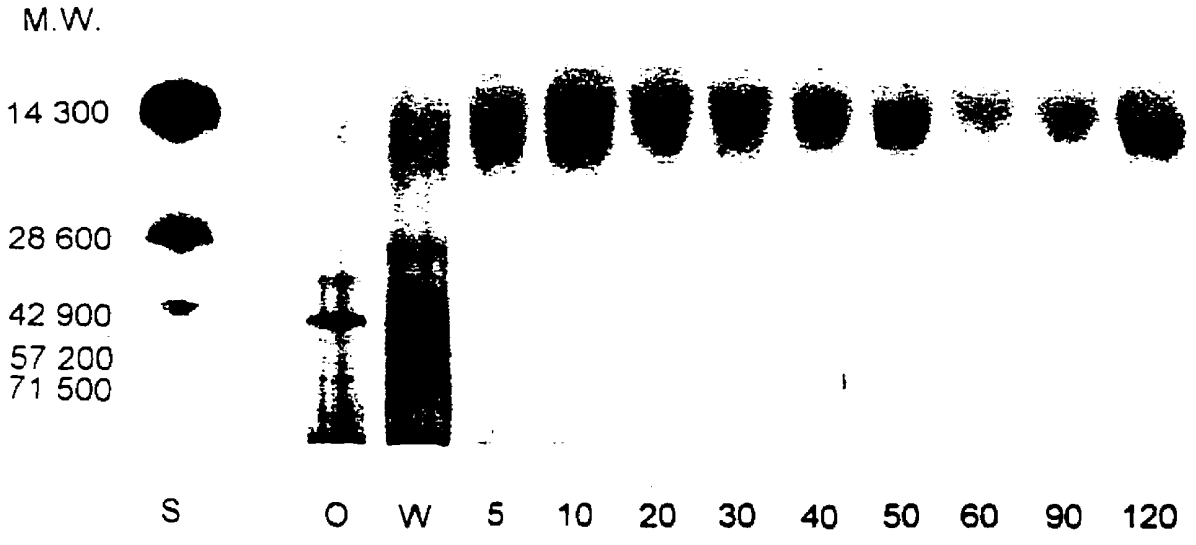

M.W.
14 300
28 600
42 900
57 200
71 500
S
O
W
5
10
20
30
40
50
60
90
120

hydrolysis, indicating that the observed electrophoretic patterns were mainly due to the action and the type of enzyme used rather than originating from the substrate type.

The hydrolysis of original shrimp protein gave bands with molecular weights of >28,600 to 71,500 Da. The water extract of the same protein, had bands corresponding to 71,500, 42,900 and <40,000 Da. Some bands with molecular weights >28,000 Da were absent in the water-extracted proteins. On hydrolysis, the disappearance of bands of molecular weights <14,300 Da was noted even at 5 min of hydrolysis using Alcalase 2.4L, an enzyme concentration of 30 AU/kg protein, a temperature of 60 °C and a meat to water ratio of 1:2 (w/v).

The protein hydrolysis patterns of lumpfish proteins on SDS-PAGE is given in Figure 4.26. The patterns are similar to those obtained for shrimp proteins. The higher molecular weight proteins in the range of >28,600 to over 71,500 Da may be either native or degradation products from myosin produced via autolysis during handling, sample preparation and storage. The medium- and high-molecular-weight peptides (>14,300 Da) were completely degraded and hence not seen on the gels after hydrolysis, as observed for shrimp protein hydrolysates. No obvious differences were observed for reaction times from 5 to 120 min used in this study. There was an increase in band intensity at molecular weight of 14,300 Da and lower, and this intensity decreased with the duration of hydrolysis.

Figure 4.26 Electrophorectrogram of lumpfish protein hydrolysis pattern. S, authentic markers; O, original lumpfish protein; W, water extracted lumpfish proteins; and hydrolysates obtained at 5, 10, 20, 30, 40, 50, 60, 90 and 120 min, respectively.


M.W.
14 300
28 600
42 900
57 200
71 500
S
O
W
5
10
20
30
40
50
60
90
120

4.7 Separation of peptide fractions using column chromatography

The elution and absorption profiles of shrimp protein hydrolysates at degrees of hydrolysis (DH) of 12.2 and 17.1% are given in Figures 4.27 and 4.28, respectively. In both hydrolysates, five fractions (I - V) were separated from shrimp protein hydrolysate using Sephadex G-15 gel filtration column chromatography based on their absorbance readings at 220 and 280 nm. The chromatographic elution and absorption pattern of hydrolysate 1 (DH=12.2%) indicates a relatively higher absorbance at 220 nm for Fractions I, II and V as compared with the corresponding fractions of hydrolysate 2 (DH=17.2%). However, similar relative absorbance values at 220 nm as compared with 280 nm were noted for both hydrolysates. Elution profile of hydrolysates was very similar to each other. There were marked differences in the relative absorbance at 220 and 280 nm for Fraction II (peak II peptide material) and this was conspicuous in elution graph of the hydrolysate. A pronounced decrease in the absorbance of peptides and aromatic amino acids was noted in Fraction V of hydrolysate 2. For all fractions which were eluted with water, the absorbance at 220 nm for the peptide bonds was greater than those at 280 nm for the aromatic rings present in the peptides (Figures 4.27 and 4.28). Fraction IV had a relatively high absorbance at 280 nm, indicating the presence of proportionally higher amounts of aromatic amino acids and other aromatic compounds in this fraction as compared with others. This fraction may also contain more amino acid residues than the other fractions. Separation of peptide fractions of sardine protein hydrolysates on the basis

Figure 4.27 Elution profile of peptides of shrimp protein hydrolysate (DH=12.2%) using Sephadex G-15 column chromatography.

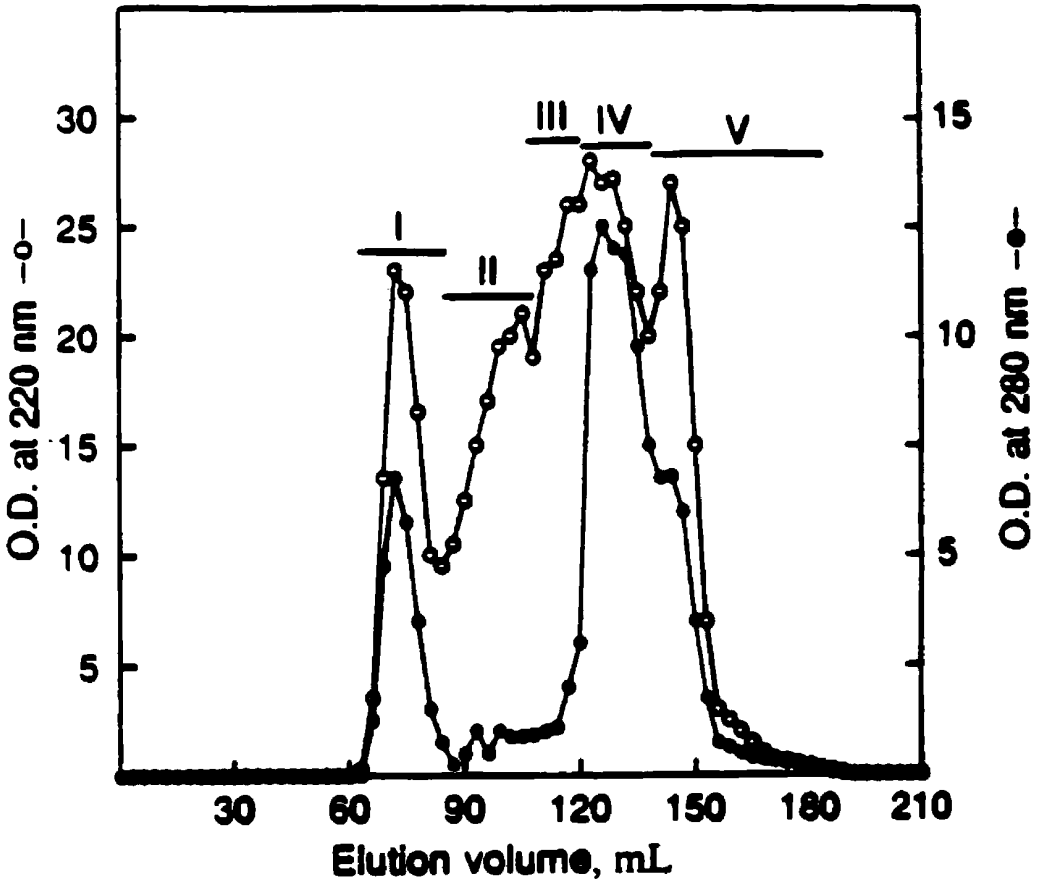
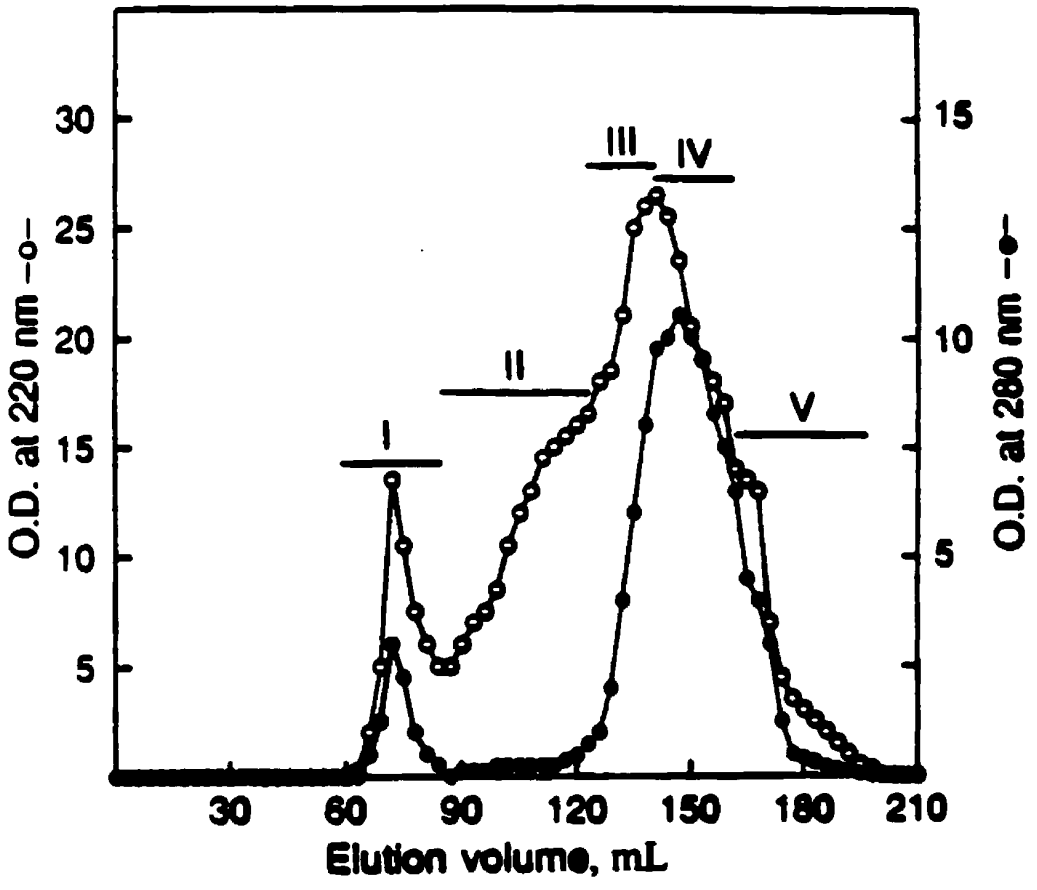

O.D. at 220 nm —o—
O.D. at 280 nm —•—
Elution volume, mL
I
II
III
IV
V
30
25
20
15
10
5
15
10
5
30
60
90
120
150
180
210

Figure 4.28 Elution profile of peptides of shrimp protein hydrolysate (DH=17.1%) using Sephadex G-15 column chromatography.


O.D. at 220 nm —o—
O.D. at 280 nm —●—
Elution volume, mL
I
II
III
IV
V
30
25
20
15
10
5
15
10
5
30
60
90
120
150
180
210

of their molecular weight distribution was achieved using Sephadex G-50 column chromatography (Quaglia and Orban, 1987a). Peptides of Alcalase-hydrolysed cod offal proteins were also separated using Sephadex G-10 column chromatography to obtain fractions based on their molecular weights (Lalasidis *et al.*, 1978). Although Alcalase has a broad specificity which may allow extensive hydrolysis, resynthesis of new peptides is possible as in the case of plastein formation where presence of free amino acids in the reaction medium leads to the formation of new peptide linkages (Fujimaki *et al.*, 1971). In general, the elution and absorption profiles of both hydrolysates indicate a good separation of peptide fractions based on their molecular weights, thus, the properties of individual fractions could be examined.

4.8 Capillary electrophoresis of peptide fractions of shrimp protein hydrolysate

The peptide fractions obtained from column chromatographic separation of shrimp protein hydrolysate (section 4.7) were subjected to capillary electrophoresis (CE). A number of resolved peaks were obtained as shown in Figures 4.29, 4.30 and 4.31. The peptide fractions (5 in total) of the hydrolysate after separation on a Sephadex gel column using water as eluent showed different electrophoretic profiles for different fractions, indicating that they differ in their composition. All five fractions contained peptides with molecular weights of <2,560 Da. Higher molecular weight peptides were also present in all fractions, however, the hydrophobic nature of these peptides played an important role in separation of different peaks observed.

Under the conditions of electrophoresis, the peptide fractions were resolved into 3 to 5 peaks. Fractions III and IV (Figure 4.30 C and D) had the highest number of peaks (5), and contained peptides with the highest molecular weights. In Fraction III, one major peak (No. 1) and four other smaller peaks were obtained. Fraction IV also had a very large peak (the major one) and four relatively smaller ones. Table 4.19 shows the molecular weights of different peaks obtained from each fraction of shrimp protein hydrolysate. The molecular weights of different fractions reflect the separation pattern of peptides in these fractions based on their molecular weights and hydrophobicity. There is, however, no distinct pattern of molecular weights for the different peaks obtained, which further establishes the point that factors other than the size of the peptides, such as degree of hydrophobicity of the peptides, may be responsible for the separation pattern observed. Using a mixture of known molecular weights of proteins, to calibrate the capillary system (Figure 4.32), molecular weights of peaks 1, 2 and 3 (elution times 13.65, 13.92 and 14.21 min, respectively) were estimated as <2,560, 2,600 and 3,200 Da, respectively. Capillary electrophoresis is a useful technique for separation of different groups of peptides of shrimp protein hydrolysates based their molecular weights and hydrophobic nature. Complimentary separations of protein subunits of peanut have been achieved by the methods of capillary and gel electrophoresis, and because of the ability of CE to separate some subunits not separable by reverse phase high performance liquid chromatography, it was concluded that the CE may still be the method of choice in some situations (Basha, 1997).

Figure 4.29 Capillary electrophorectograms of lyophilized shrimp protein hydrolysate fractions (I and II) obtained from Sephadex gel column chromatography (A, Fraction I; B, Fraction II).

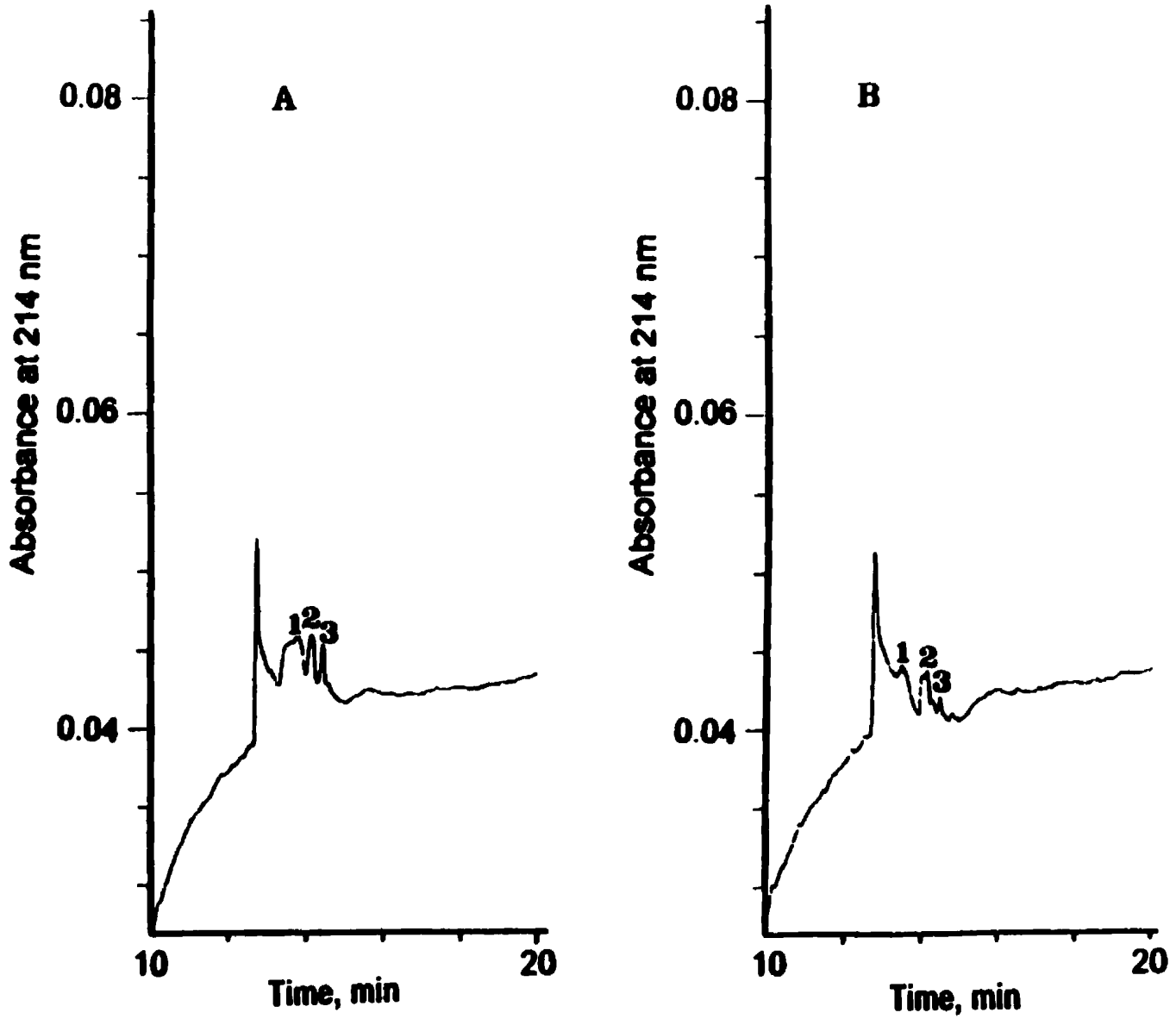

A
B
0.08
0.06
0.04
Absorbance at 214 nm
1
2
3
10
20
Time, min

Figure 4.30 Capillary electrophorectograms of lyophilized shrimp protein hydrolysates fractions (III and IV) obtained from Sephadex gel column chromatography (C, Fraction III; D, Fraction IV).

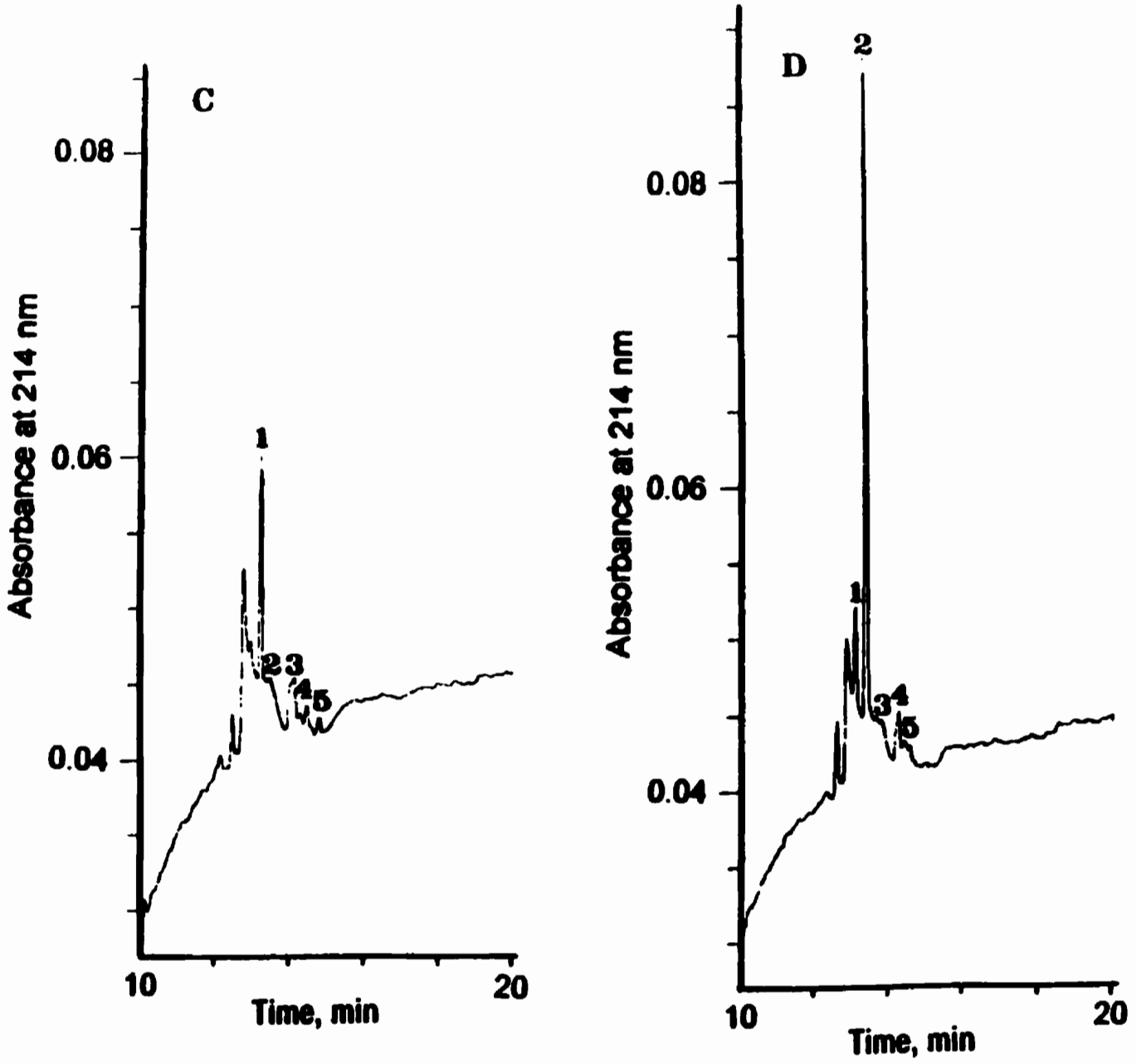

C
D
0.08
0.06
0.04
Absorbance at 214 nm
1
2
3
4
5
10
20
Time, min

Figure 4.31 Capillary electrophorectogram of lyophilized shrimp protein hydrolysate fraction (V) obtained from Sephadex gel column chromatography (E. Fraction V).

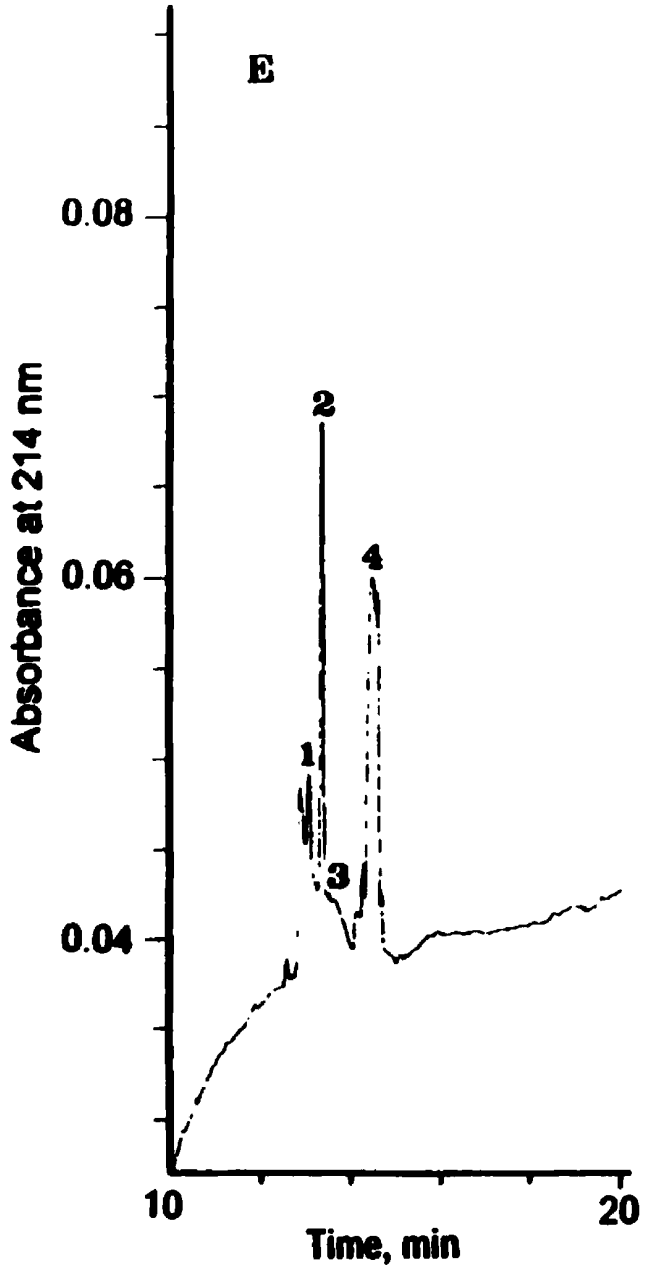
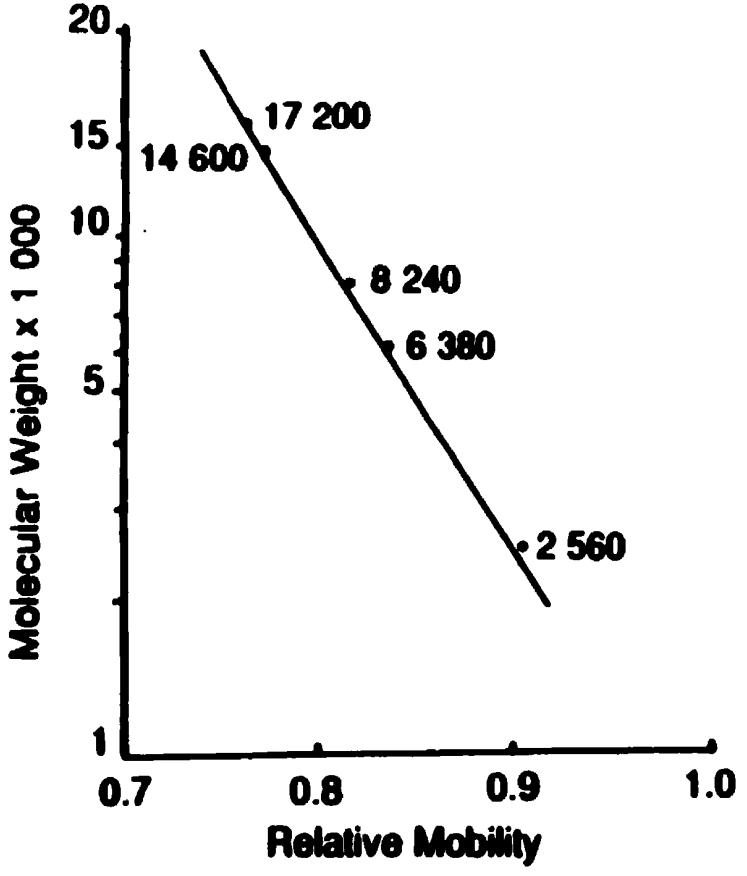
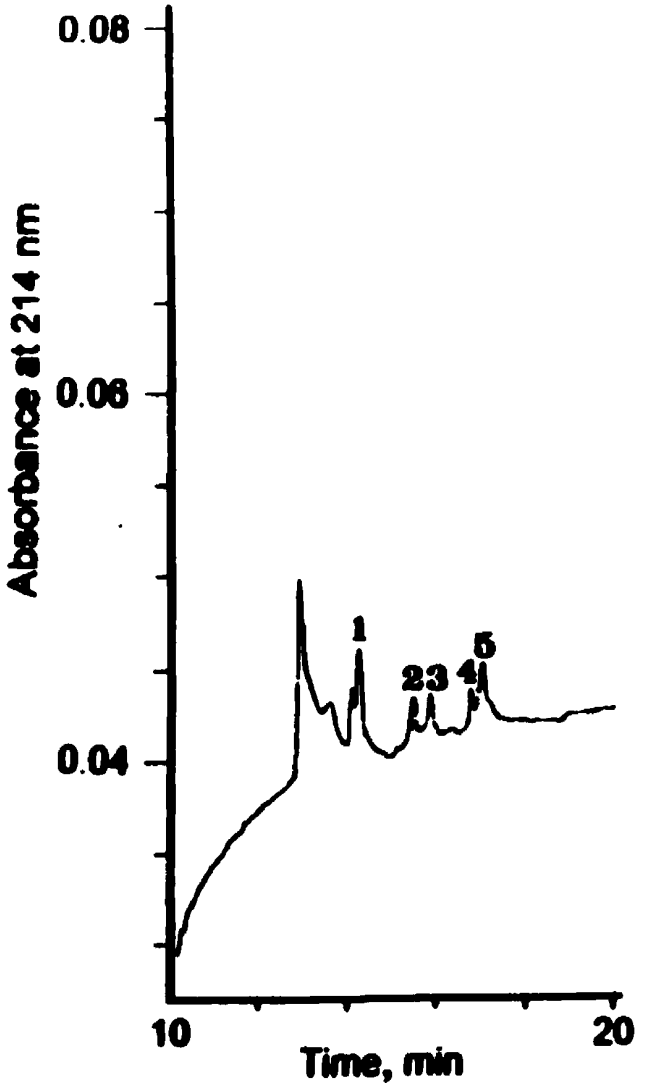

E
0.08
0.06
0.04
Absorbance at 214 nm
1
2
3
4
10
20
Time, min

Table 4.19 Molecular weights of different peaks obtained from capillary electrophoresis of the different peptide fractions of shrimp protein hydrolysate.

Fraction	Peak	Molecular weight
I	1	<2560
	2	2600
	3	3200
II	1	<2560
	2	2600
	3	3200
III	1	<2560
	2	<2560
	3	3200
	4	4200
	5	6000
IV	1	<2560
	2	<2560
	3	3200
	4	3900
	5	4500
V	1	<2560
	2	<2560
	3	<2560
	4	2800

Figure 4.32 Calibration graph and electrophorectogram of standard proteins used for capillary electrophoresis.


Molecular Weight x 1 000
20
15
10
5
1
17 200
14 600
8 240
6 380
2 560
0.7
0.8
0.9
1.0
Relative Mobility

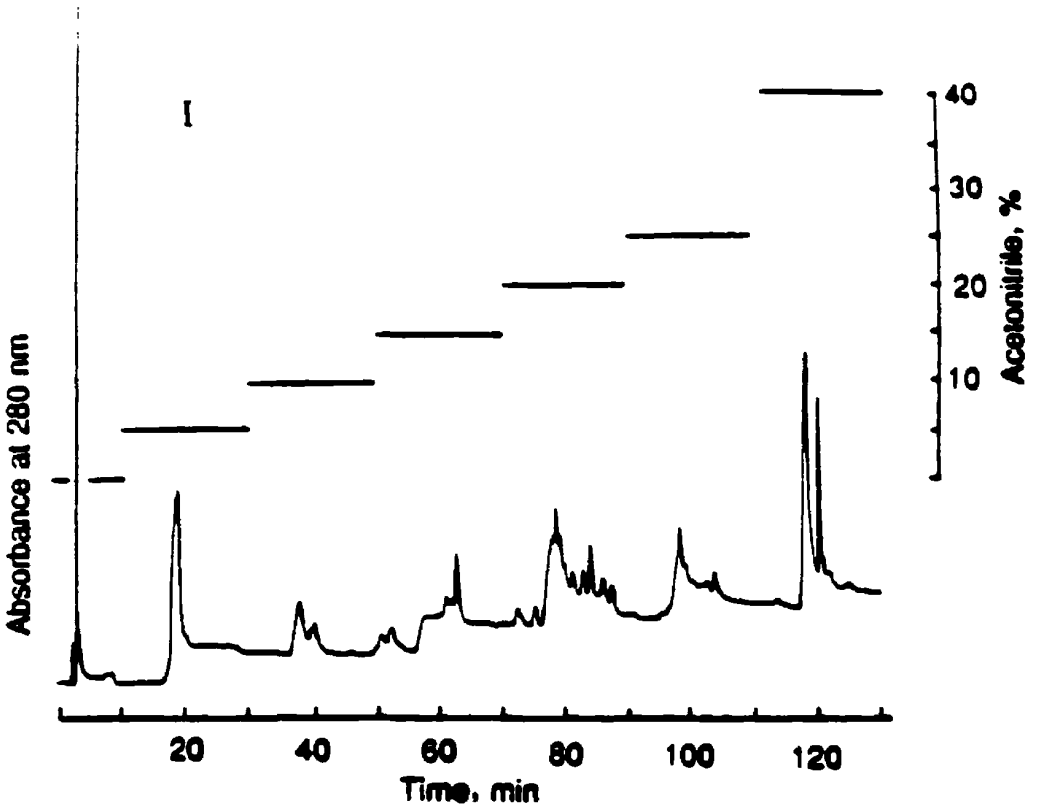

Absorbance at 214 nm
0.08
0.06
0.04
1
2
3
4
5
10
20
Time, min

4.9 High performance liquid chromatographic (HPLC) analysis of peptide fractions

The HPLC in the reverse mode allowed separation of mixtures of peptides present in Fractions I to V, obtained via Sephadex G-15 column chromatography. The use of HPLC column packed with C18 group and stepwise gradient concentration of acetonitrile-water-trifluoroacetic acid as solvent, resulted in separation of groups of peptides as indicated by their multipeak profile (Figures 4.33-4.35). The separation of the peptides was based on their hydrophillic characteristics. The order of elution of peptides was from the most hydrophillic to the least hydrophillic. The use of HPLC in conjunction with reverse-phase bonded silica support is known to considerably facilitate the separation and purification of peptides (Rivier, 1978; Schroeder *et al.*, 1979; Kratzin and Yang, 1981). Reverse phase HPLC involves hydrophillic interaction, thus one of the criteria for resolution is that the hydrophobic moieties of peptide molecules interact specifically with the bonded phase. The octadecyl group used influences the strength of such interactions.

At time zero, when water with 0.5% trifluoroacetic acid was used, the most hydrophillic peptides eluted first. When acetonitrile was used and its concentration increased stepwise, with a corresponding increase in hydrophobic nature of the solvents, more hydrophobic peptides were eluted (Figure 4.33). These characteristic peptide patterns may be due to the dominance of polar and nonpolar amino acids in the peaks that eluted first and later, repsectively. The HPLC elution patterns of

Figure 4.33 Chromatogram of peptide Fractions I and II of shrimp protein hydrolysate in a reversed-phase HPLC.


I
Absorbance at 280 nm
Acetonitrile, %
40
30
20
10
20
40
60
80
100
120
Time, min

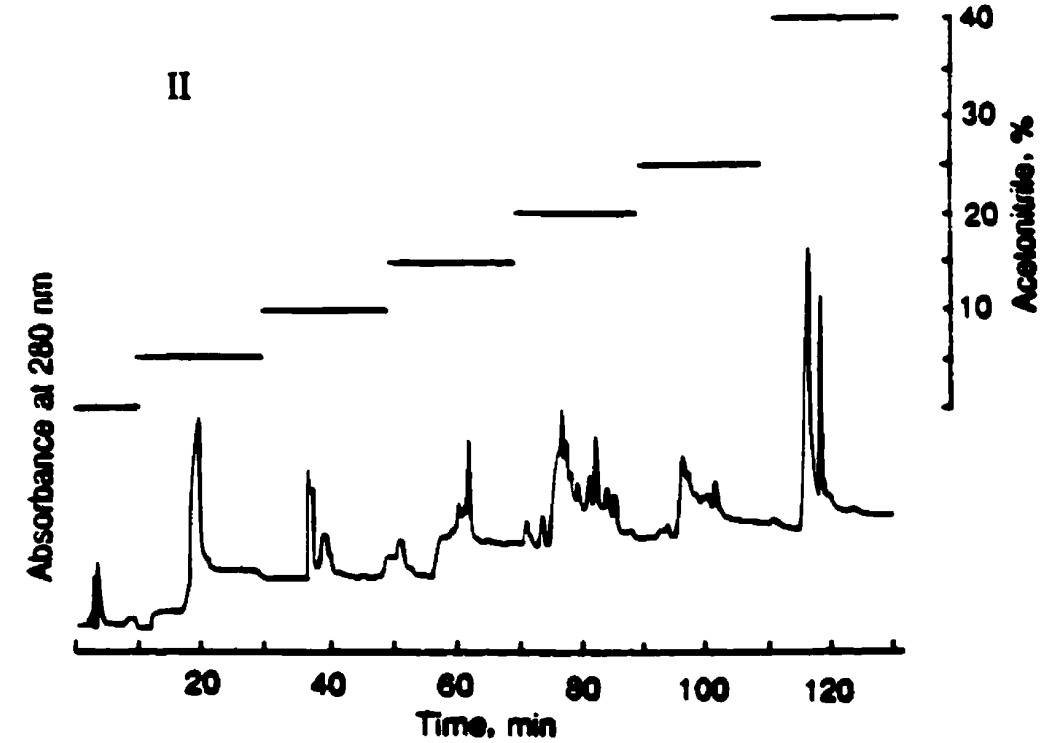
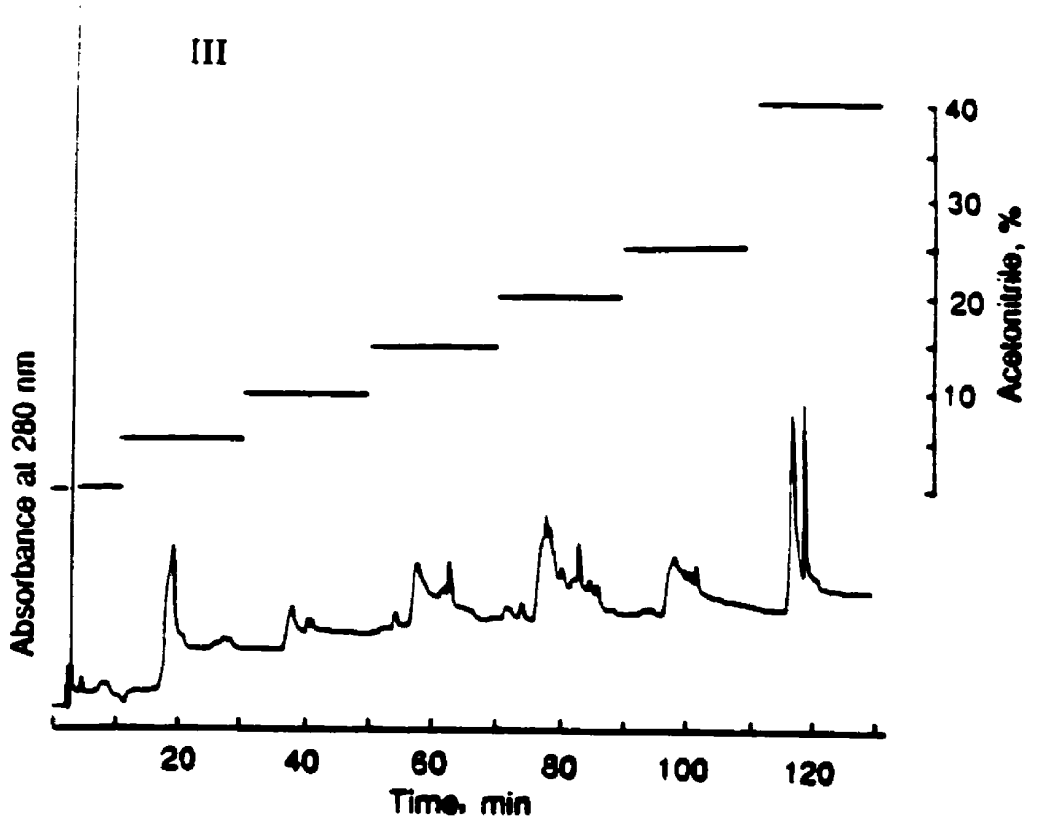

II
Absorbance at 280 nm
Acetonitrile, %
40
30
20
10
20
40
60
80
100
120
Time, min

Figure 4.34 Chromatogram of peptide Fractions III and IV of shrimp protein hydrolysate in a reversed-phase HPLC.


III
Absorbance at 280 nm
Acetonitrile, %
40
30
20
10
20
40
60
80
100
120
Time, min

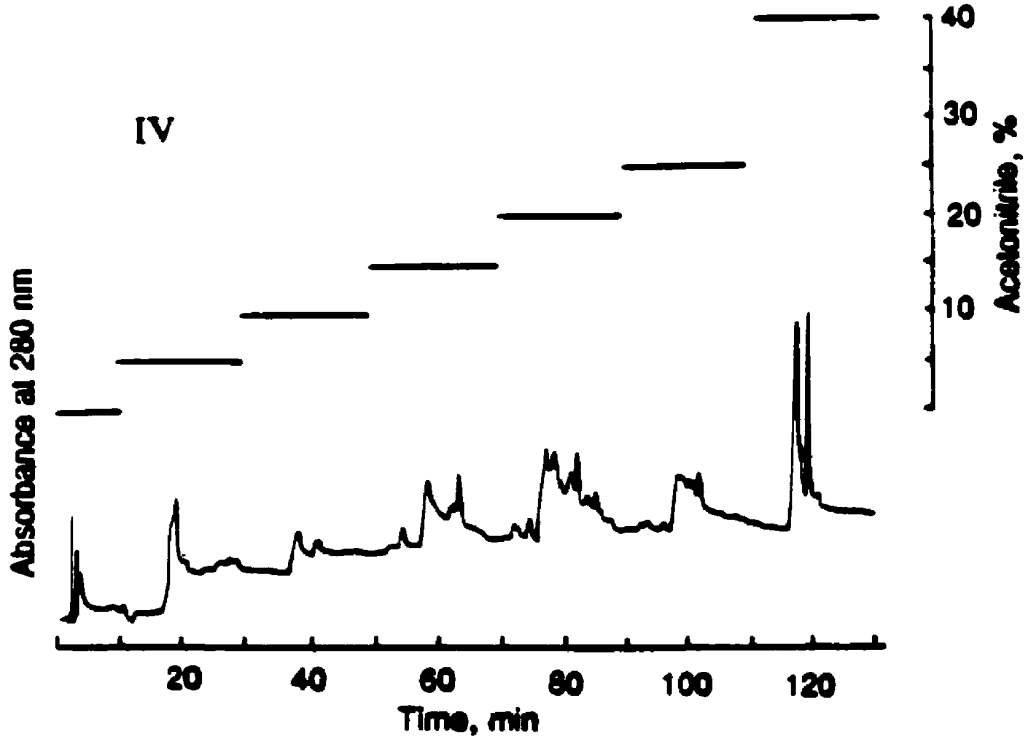
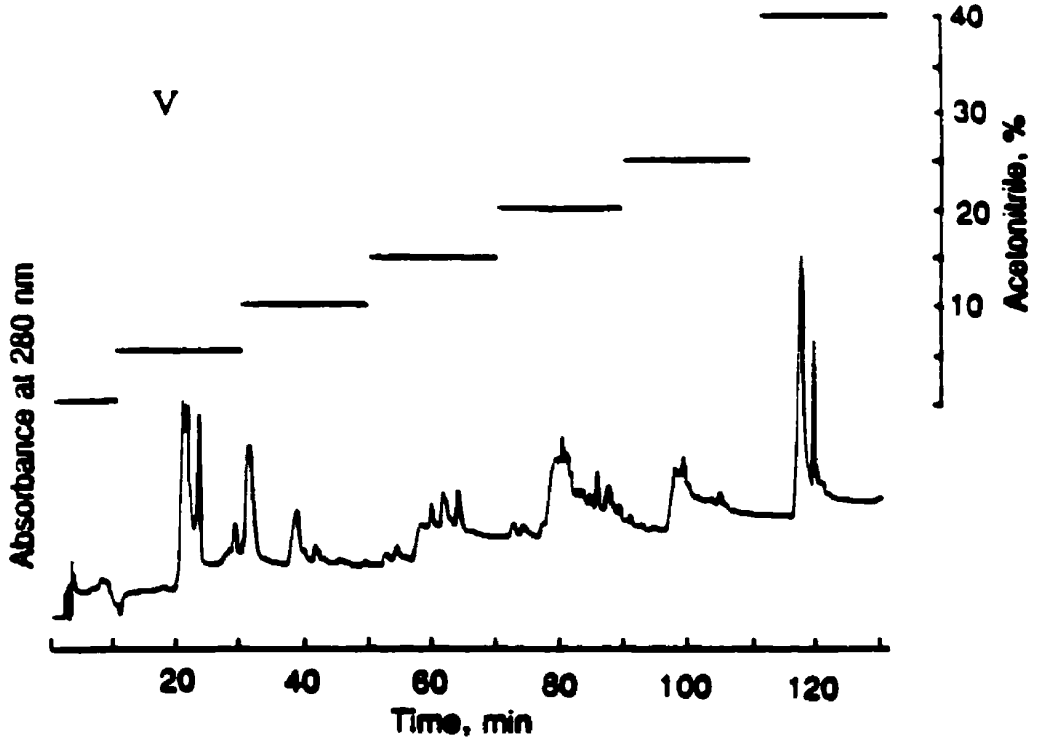

IV
Absorbance at 280 nm
Acetonitrile, %
40
30
20
10
20
40
60
80
100
120
Time, min

Figure 4.35 Chromatogram of peptide Fraction V of shrimp protein hydrolysate in a reversed-phase HPLC.


V
Absorbance at 280 nm
Acetonitrile, %
40
30
20
10
20
40
60
80
100
120
Time, min

peptides of chicken, pork and beef hydrolysates were characterized by three major peaks due to the presence of more polar fractions as compared with one major peak in soy hydrolysate due to the presence of less polar fractions (Medina and Phillips, 1982). The peptides obtained during the first 40 min of separation were of sharper and better defined peaks, indicating good resolution. Adequate peak symmetry was also found at the end of the gradient. A similar profile was noted in the separation of hydrolysate of monoclonal L-chain of κ-type protein (Kratzin and Yang, 1981). The trypsin digest of this protein (MW 23,000) was separated on a Zobax C_{18} reverse phase column (250 x 4.6 mm) in 40 min. The total time required for separation of each of the five shrimp peptide fractions was 120 min.

Although some of the peaks have smooth shape, they often contain more than one peptide, thus interpretation of chromatograms like those in Figures 4.33-4.35 is difficult. However, it may suffice to say that the peptides of shrimp head hydrolysates have been separated on the basis of their hydrophobicity. The gradient elution of peptides using acetonitrile and water containing trifluoroacetic acid (TFA) was performed as acetonitrile is transparent and clear and is detected at a low wavelength of 220 nm, thus allowing observation of all peptides because of the significant end absorption of the peptide bonds at this wavelength. The peptide Fraction IV was found to have seven components. The corresponding elution profile for the peptides in this fraction is shown in Figure 4.34. The resolving power of reverse phase system is excellent with major components resolved within 40 min gradient. A linear

gradient of 5 to 20% (v/v) acetonitrile was used with 0.1% phosphoric acid to obtain 11 major components in a 15-min gradient for trypsin digest of Actinidin (Hancock *et al.*, 1978).

4.10 Antioxidant activity of protein hydrolysates

4.10.1 Antioxidant activity in a β-carotene/linoleate emulsion system

Shrimp and lumpfish hydrolysates, when assessed in a β-carotene/linoleate emulsion system, both delayed the bleaching of the β-carotene, thus indicating their antioxidative activity. Such activity was concentration-dependent, with the 5 mg freeze-dried shrimp protein hydrolysate having less effect than 10 mg protein hydrolysate in the test solution (Figure 4.36). The lumpfish hydrolysate also performed in a similar manner in the emulsion system, however, it was less effective than shrimp hydrolysate in retarding the bleaching of β-carotene.

4.10.2 Antioxidant activity of protein hydrolysate in a meat model system

In addition to the antioxidant activity of protein hydrolysates noted in a β-carotene/linoleate system, the hydrolysates also inhibited the formation of 2-thiobarbituric acid reactive substances (TBARS) in a meat (pork) model system (Figure 4.37). Since antioxidant activity is often system-dependent, it is better to assess them in more than one system because of their potential use in different food formulations. Antioxidant activity is exerted via different mechanisms based on the

Figure 4.36 Antioxidant activity of shrimp protein hydrolysates in a β-carotene/linoleate model system, as measured by changes in absorbance values at 470 nm.

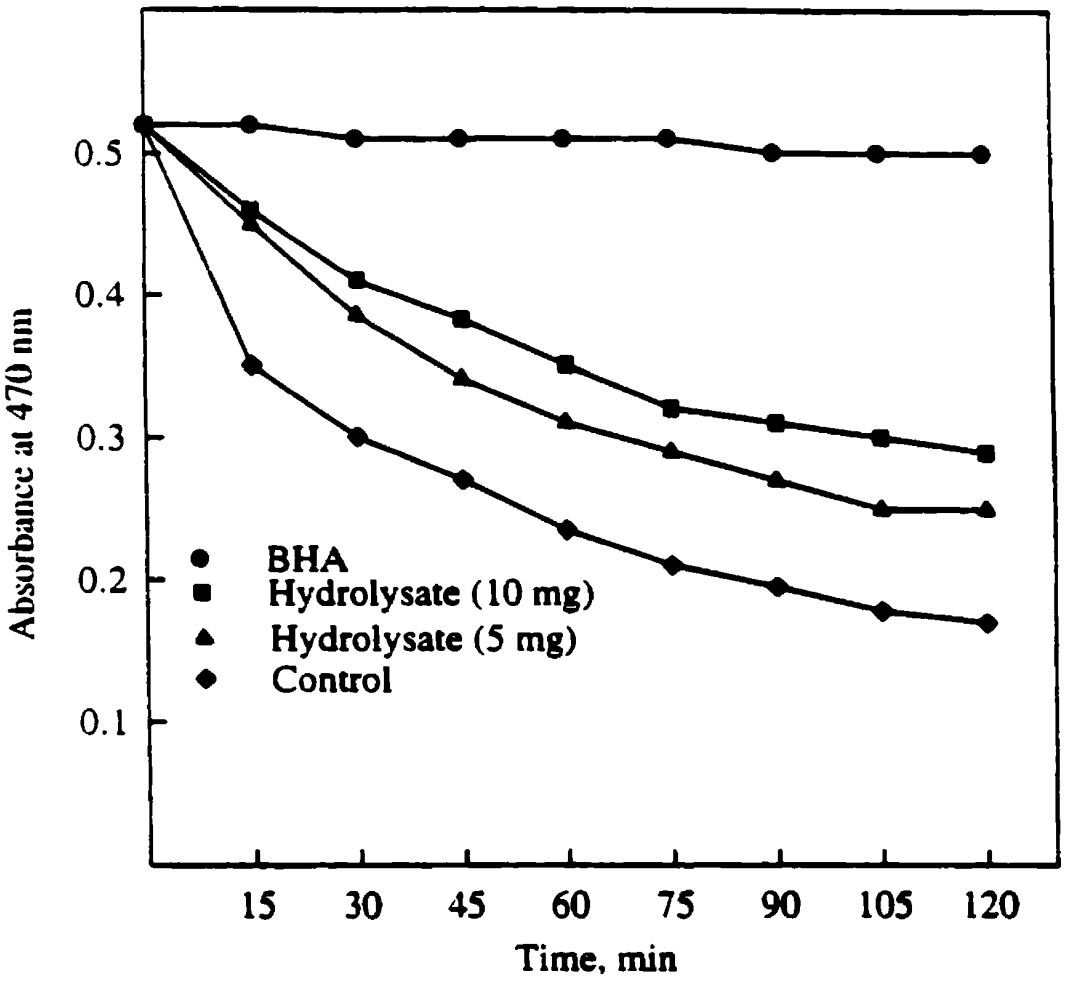
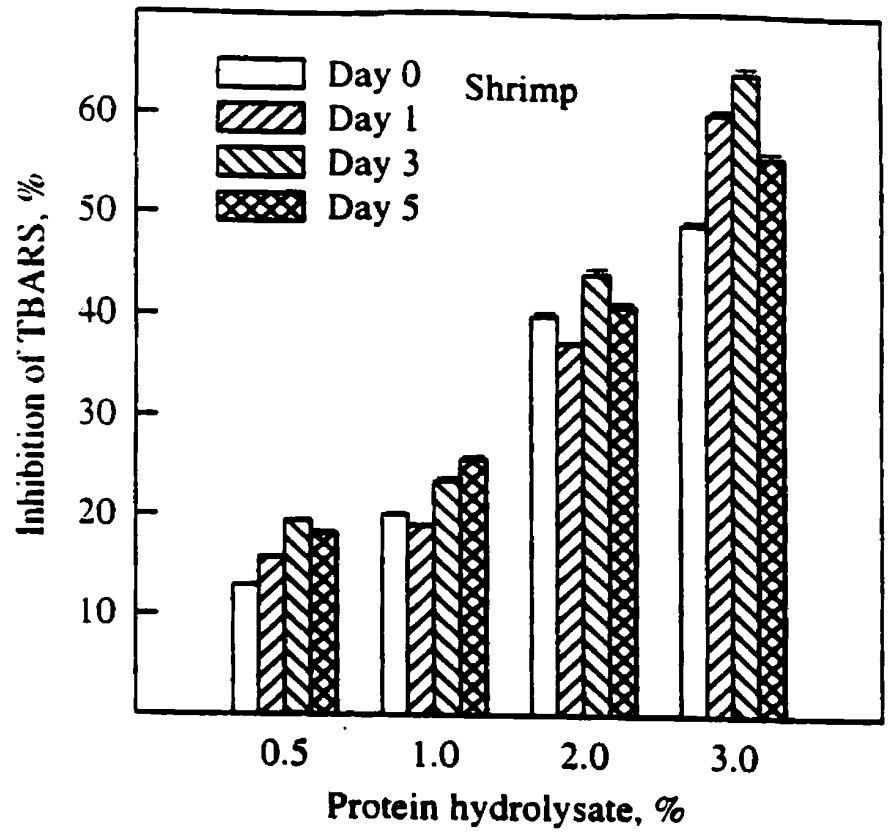
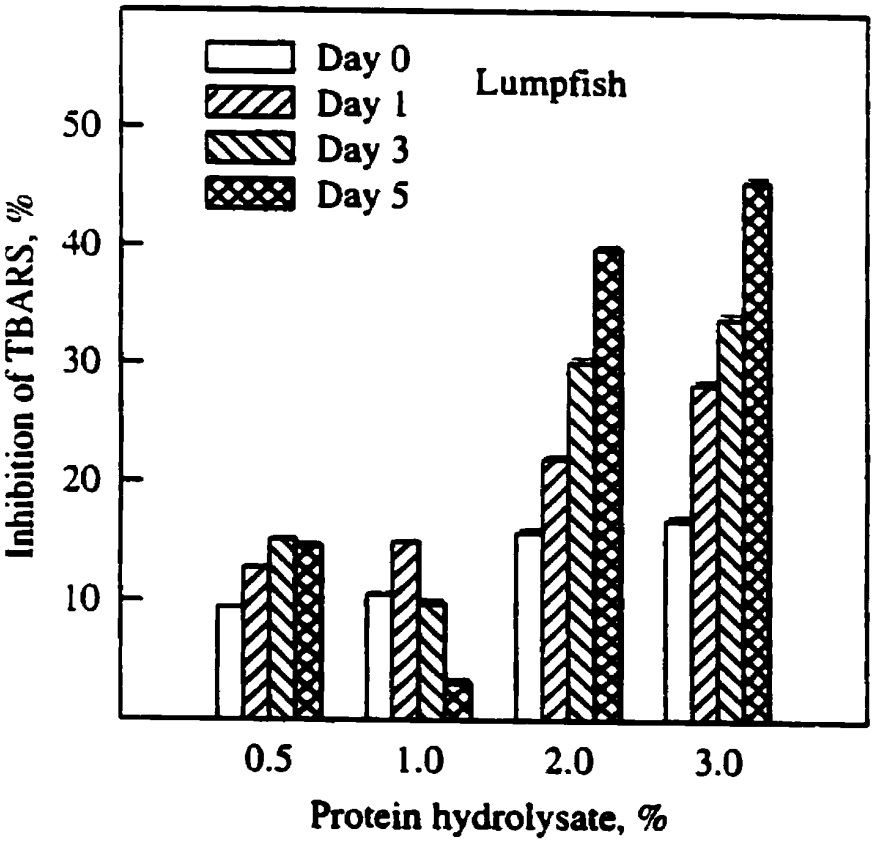

Absorbance at 470 nm
0.5
0.4
0.3
0.2
0.1
BHA
Hydrolysate (10 mg)
Hydrolysate (5 mg)
Control
15
30
45
60
75
90
105
120
Time, min

Figure 4.37 Inhibition of formation of 2-thiobarbituric acid reactive substances (TBARS) by shrimp (top) and lumpfish (bottom) protein hydrolysates in cooked meat system stored at 4 °C.


Day 0
Day 1
Day 3
Day 5
Shrimp
Inhibition of TBARS, %
60
50
40
30
20
10
0.5
1.0
2.0
3.0
Protein hydrolysate, %


Day 0
Day 1
Day 3
Day 5
Lumpfish
Inhibition of TBARS, %
50
40
30
20
10
0.5
1.0
2.0
3.0
Protein hydrolysate, %

type of system in which they are tested (Frankel *et al.*, 1996). Pryor *et al.* (1988) concluded from their experiments, using different systems, that the rate constants for antioxidants are very sensitive to the system used for measurements. Thus, rates obtained with a micellar system did not parallel those obtained for homogenous systems.

The protein hydrolysates, as such, when added to cooked ground pork and stored at refrigeration temperature, retarded TBARS formation. The effectiveness of shrimp protein hydrolysates in this regard was concentration-dependent. Thus, the effect was highest at 3.0% (w/w) and lowest at 0.5% (w/w) during a 5-day refrigerated storage period. The inhibition of TBARS formation ranged from 12.9% at 0.5% hydrolysate immediately after cooking to 64.2% at 3.0% hydrolysate in the meat system on day 3 (Figure 4.37). The actual TBARS numbers did not exceed 2.3 mg/kg sample in any of the samples examined. Capelin protein hydrolysates have been reported to display antioxidant activity in a meat model system by inhibiting TBARS formation (Shahidi *et al.*, 1995).

The lumpfish protein hydrolysates were also effective in inhibiting TBARS formation in a meat system at 0.5-3.0% (w/w) [Figure 4.37]. However, lumpfish hydrolysates were less effective as compared to their shrimp counterparts. A 45.6% inhibition of TBARS formation was observed in a meat model system upon the addition of 3.0% (w/w) lumpfish hydrolysate. The observed differences in the degree

of retardation of lipid oxidation in the presence of shrimp and lumpfish protein hydrolysates may be due to the composition and types of peptides present in both preparations, as well as possible presence of antioxidant (non-peptide) phenolic compounds in shrimp hydrolysates. A combination of these factors may be responsible for the more effective antioxidant performance of shrimp hydrolysate as compared with that of lumpfish.

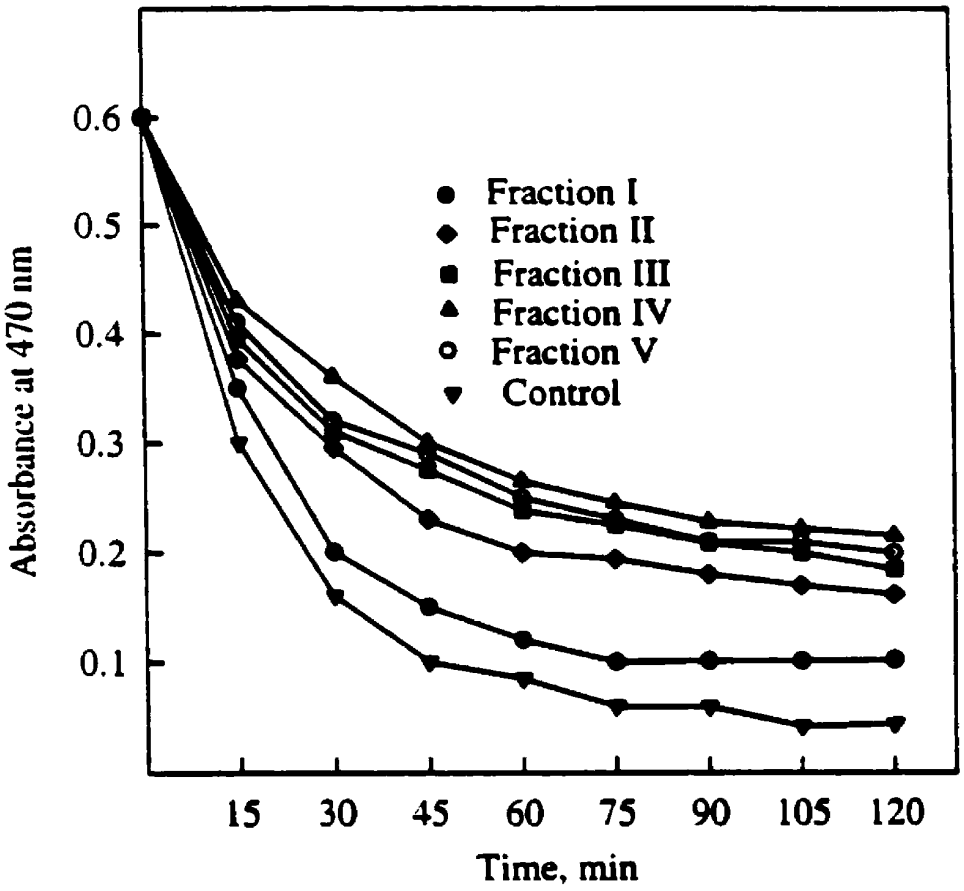
The 2-thiobarbituric acid (TBA) test is commonly used for determining the rate of lipid oxidation in muscle foods. This test measures mainly aldehydes as part of TBARS formed during oxidation of lipids. These aldehydes are responsible for the off-flavour notes of oxidized lipid-containing foods (King *et al.*, 1993). The TBARS content of cooked meat products showed good correlation with the sensory scores (Zipser *et al.*, 1964; Shahidi *et al.*, 1987; St. Angelo *et al.*, 1990). Inhibition of TBARS formation in the presence of protein hydrolysates, is also expected to retard the formation of off-flavours associated with lipid oxidation in foods.

Hydrolysed soy proteins have been shown to possess antioxidant activity in lard (Pratt and Birac, 1979). Bishov and Henick (1975) evaluated soy protein and autolysed yeast protein in a freeze-dried model system consisting of the hydrolysates and corn oil. Low-molecular-weight peptides, less than 700 Da, had a greater effect as primary antioxidants than high-molecular-weight proteins.

4.11 Antioxidant activity of peptide fractions of shrimp protein hydrolysates in a β-carotene/linoleate model system

The five fractions separated from shrimp hydrolysate with DH=12.2% (hydrolysate 1) using a Sephadex column exhibited antioxidant activity in a β-carotene/linoleate model system (Figure 4.38). Fractions III, IV and V exhibited the highest degree of antioxidant activity by delaying the bleaching of β-carotene over a 120-min period. Although fractions I and II possessed antioxidant properties, their activity was less pronounced in this system. Differences in antioxidant properties of peptide fractions noted in this study might originate from existing differences in the composition of different peptides. The presence of certain amino acids such as histidine in a peptide has been reported to be essential for antioxidant activity (Chen *et al.*, 1995). The sequence of amino acids in the peptide chain may also play a role in the antioxidant activity exhibited by a particular peptide. The type of terminal amino acid in dipeptides has been reported to also influence the antioxidant activity observed, as N-terminal branched chain amino acid was preferred over a C-terminal one for antioxidant activity (Kawashima *et al.*, 1979). Studies have also shown that certain peptides exhibit antioxidant activity whereas their constituent amino acids, when used individually or in combination, do not (Decker *et al.*, 1992; Chan *et al.*, 1994). This suggests that the peptide linkage between the amino acid moieties of peptides may be involved in the antioxidant activity. The synergistic action of the peptides with the emulsifier (Tween 40) used in the analysis or with other peptides in

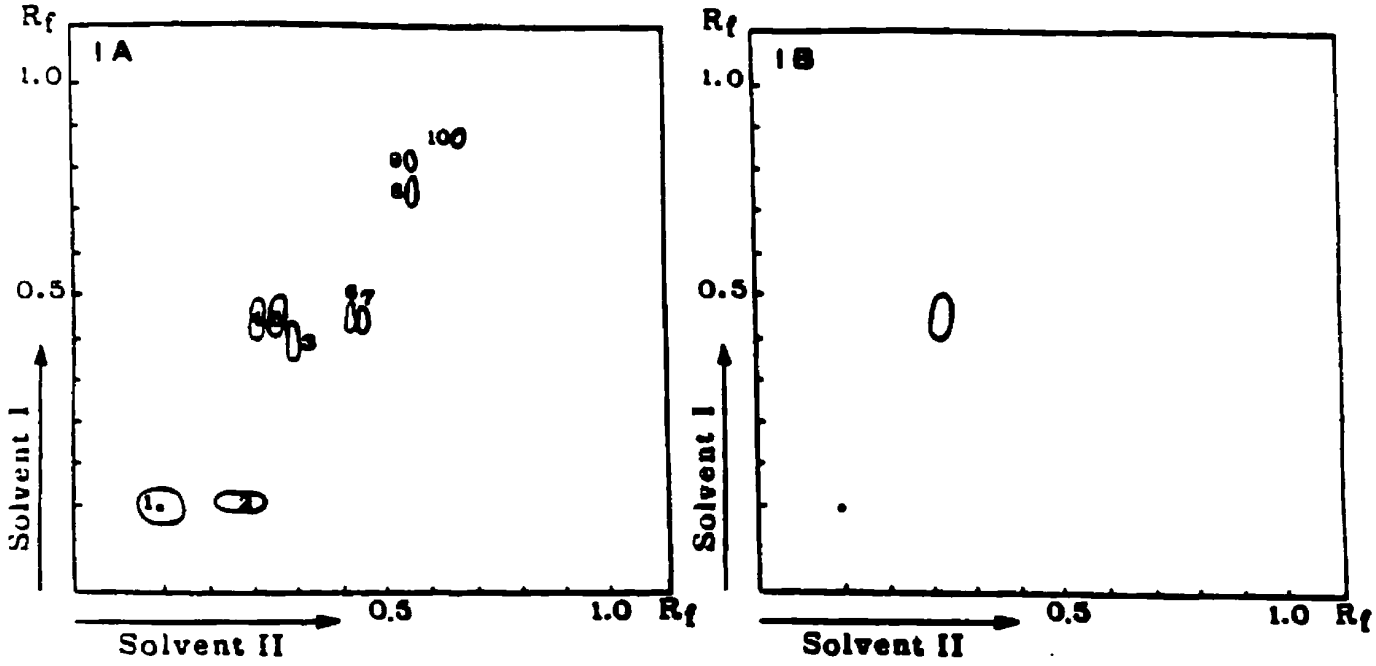
Figure 4.38 Antioxidant activity of peptide fractions of shrimp protein hydrolysates in a β-carotene-linoleate model system, as measured by changes in absorbance values at 470 nm.


Absorbance at 470 nm
0.6
0.5
0.4
0.3
0.2
0.1
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Control
15
30
45
60
75
90
105
120
Time, min

the system could have also contributed to the observed differences in the antioxidant activity of peptides. Emulsifiers are known to decrease the particle size of peptides and increase the contact surface of the phases involved (Marcuse, 1962).

Thin layer chromatographic analysis of the hydrolysate of shrimp protein resulted in a number of spots when the plates were sprayed with a 0.2% ninhydrin in acetone. (Figures 4.39-4.41). These indicated the presence of peptides and amino acids. The number of spots obtained varied from one fraction to another. While fraction IV had the highest number, 19 spots (Figure 4.41), fraction II had the least, with only 6 spots (Figure 4.39). Upon spraying of another set of TLC plates with a β-carotene/linoleate solution the presence of only antioxidant compounds which retained the yellow colour spots without complete bleaching of the plates overnight was detected. The majority of the antioxidant compounds were detected in fraction IV (Figure 4.40), however, other fractions (I, II, III and V) also had spots indicating antioxidant activity. The presence of most spots which exhibited antioxidant activity was noted in fraction IV, which supports the results of the β-carotene/linoleate experiments reported in the preceding section. Some of the spots with antioxidant activity in Figures 4.39B, 4.40B, and 4.41B were not present on the plates sprayed with ninhydrin solution; this may suggest the presence of antioxidant compounds in the hydrolysates other than the peptides or amino acids. The presence of antioxidant peptides in shrimp hydrolysates provides further support to the previously reported results regarding the presence of antioxidant peptides in seal protein hydrolysate

Figure 4.39 Two-dimensional thin layer chromatography of peptide fractions I and II of shrimp protein hydrolysate; plates were sprayed with: A, ninhydrin solution; B, a β-carotene-linoleate solution.


I A
I B
Rf
1.0
0.5
Solvent I
Solvent II
0.5
1.0 Rf

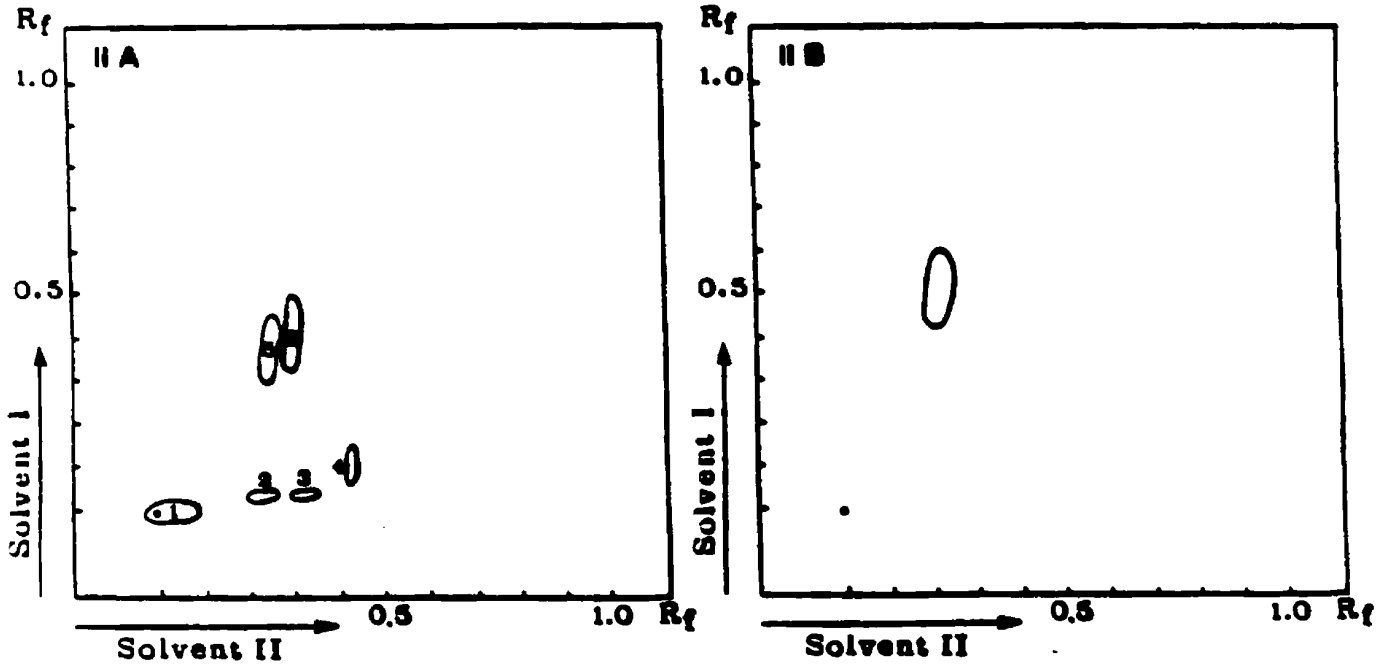
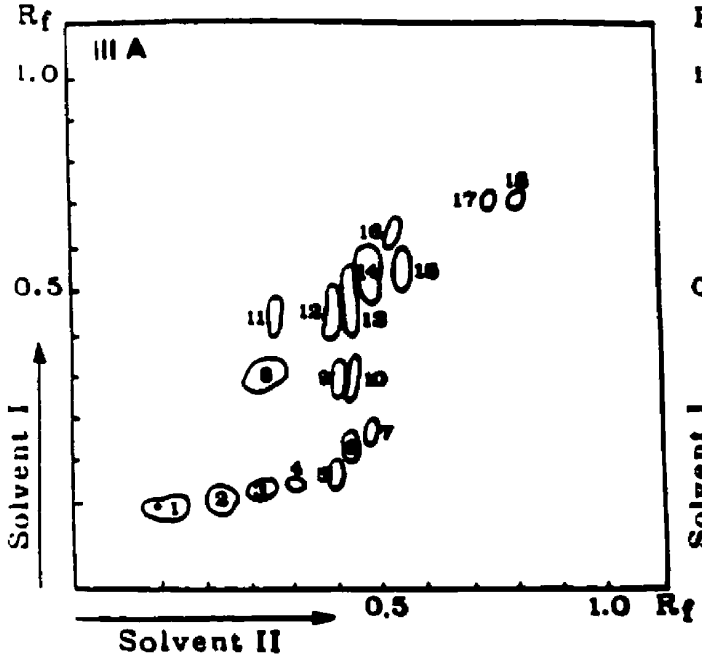
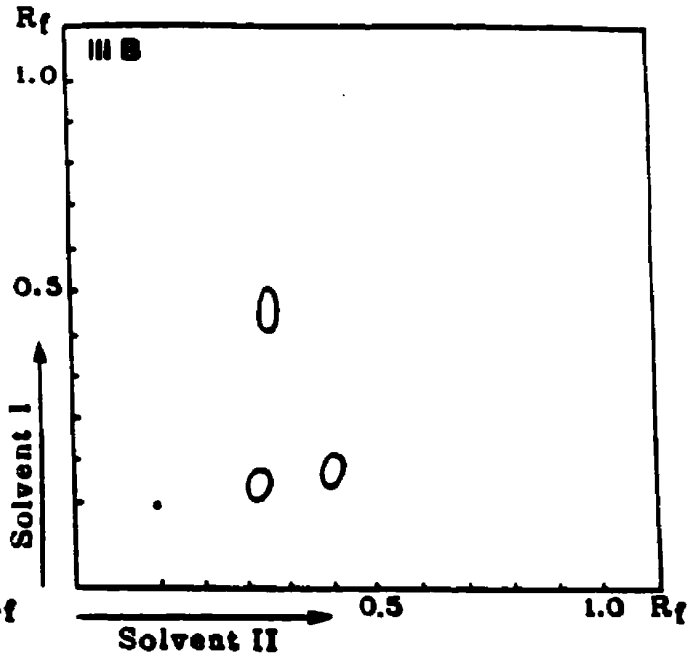
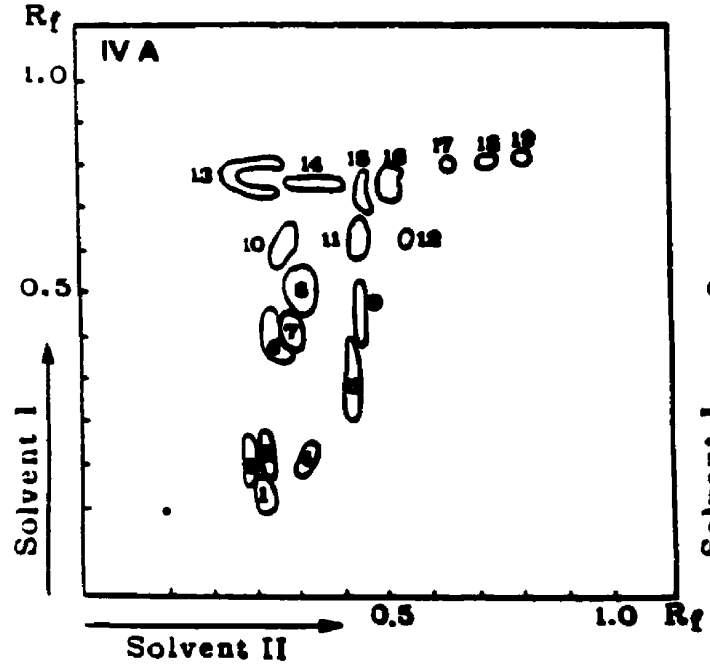

II A
II B
Rf
1.0
0.5
Solvent I
Solvent II
0.5
1.0 Rf

Figure 4.40 Two-dimensional thin layer chromatography of peptide fractions III and IV of shrimp protein hydrolysate; plates were sprayed with: A. ninhydrin solution; B. a β-carotene-linoleate solution.


III A
R_f
1.0
0.5
Solvent I
Solvent II
0.5
1.0 R_f


III B
R_f
1.0
0.5
Solvent I
Solvent II
0.5
1.0 R_f


IV A
R_f
1.0
0.5
Solvent I
Solvent II
0.5
1.0 R_f

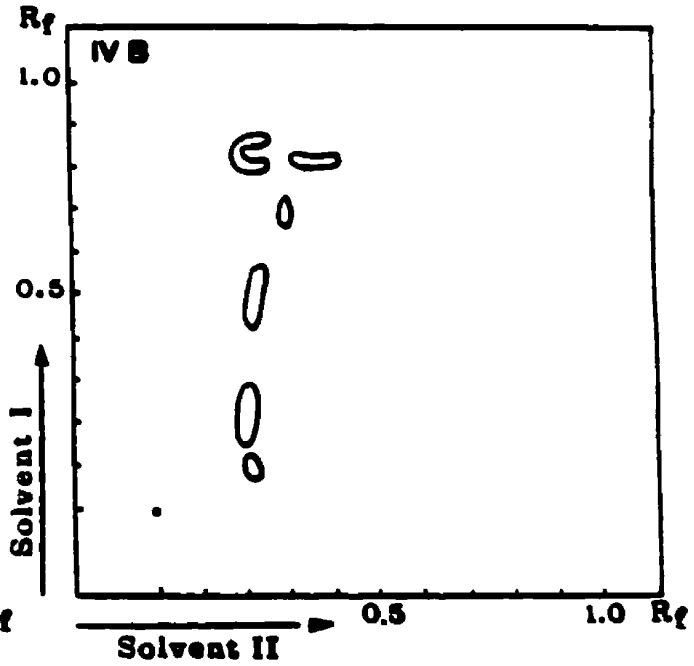
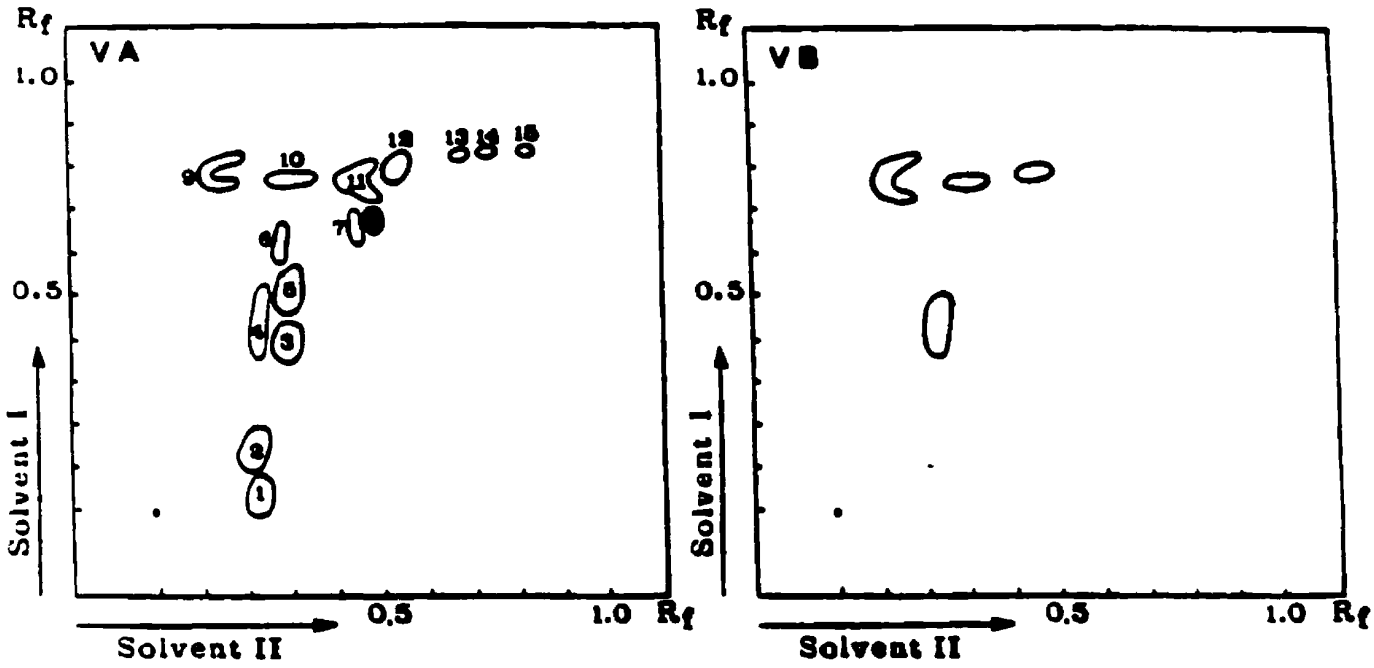

IV B
R_f
1.0
0.5
Solvent I
Solvent II
0.5
1.0 R_f

Figure 4.41 Two-dimensional thin layer chromatography of peptide fractions V of shrimp protein hydrolysate; plates were sprayed with: A. ninhydrin solution; B. a β-carotene-linoleate solution.


Rf
1.0
0.5
VA
Solvent I
0.5
1.0 Rf
Solvent II
Rf
1.0
0.5
VB
Solvent I
0.5
1.0 Rf
Solvent II

(Shahidi and Amarowicz, 1996). However, the presence of prooxidant peptides was also reported in seal protein hydrolysates. Furthermore, six antioxidant peptides have been identified in the hydrolysate of conglycinin, a soybean protein (Chen *et al.*, 1995). The antioxidant activity of certain imidazole dipeptides has been extensively studied (Chan and Decker, 1994). The dipeptides, including carnosine, anserine and ophidine, are believed to exert their antioxidant activity by metal chelation or free radical scavenging (Kohen *et al.*, 1988; Decker *et al.*, 1992).

4.12 Thermally generated flavour compounds from shrimp hydrolysates and glucose

The effects of enzymatic hydrolysis and degree of hydrolysis on the formation of volatile flavour compounds via interaction with glucose were examined. Many flavour compounds were generated due to the thermal reaction between glucose and shrimp proteins, as such, or its hydrolysates at different degrees of hydrolysis. These include pyridines, pyrazines, aldehydes, furan derivatives, sulphur-containing compound, among others. As shown in Table 4.20 over 50 thermally-generated flavour compounds were identified. The number of major volatiles and carbonyl compounds generated are given in Table 4.21. The concentration of 6 pyrazines detected in the thermal reaction of shrimp protein and glucose increased after enzymatic hydrolysis. However, no pronounced increases were observed for other compounds identified (Table 4.21). In general, the number and concentration of

Table 4.20 Flavour compounds identified and quantified from thermal reaction of shrimp protein or its hydrolysates with glucose.

Compounds	Concentration (ppm)		
	Shrimp protein + glucose	Hydrolysate 1 + glucose	Hydrolysate 2 + glucose
Pyrazine	17	trace	43
Methylpyrazine	90	118	134
2,5-dimethylpyrazine	201	330	332
2,3-dimethylpyrazine	16	16	16
Trimethylpyrazine	72	124	123
3-ethyl-2,5-dimethylpyrazine	2	11	13
2-acetylpyridine	7	ND	ND
4-(1-pyrrolidinyl)-pyridine	13	ND	ND
3-isopropyl-2, 4, 5-trimethylpyridine	12	ND	ND
Pyrrolidine	ND	8	ND
2-furfural	34	44	45
2-acetylfuran	7	ND	ND
5-methyl-2-furfural	trace	10	10
5-(Hydroxymethyl)2-furfural	ND	10	4
2(5H) Furanone	4	ND	ND
2,5-dimethyl-3(2H)-furanone	8	ND	ND
Dihydro-2-methyl-3(2H)-furanone	7	14	17
3-methoxy-5-methyl-2(5H)-furanone	5	ND	ND
2-furanmethanol	76	55	52

Table 4.20 continues .....

1H-indole	ND	ND	6
9H-pyrido[3,4-b]indole	ND	9	ND
2,5-dimethyl-1-propyl-pyrrole	9	ND	9
N-N-dimethyl phenylethylamine	40	31	27
N-(4-formylphenyl)-acetamide	ND	12	11
Maltoxazine	20	24	ND
Hexahydro-1,3,5-trimethyl-1,3,5-triazine	ND	ND	31
1-methyl-2-pyrrole carboyaldehyde	6	ND	ND
2-(acetylamino)-2-propenoic acid	ND	17	ND
Cyclo(L-phenylalanyl-L-prolyl)	22	58	69
Cyclo(L-Leucyl-L-phenylalanyl)	ND	98	122
Dimethyl disulphide	5	13	15
1-hydroxy-2-propanone	124	78	83
3-Methylbutanal	107	516	639
2-Methylbutanal	ND	97	120
Pentanal	3	ND	ND
Hexanal	6	ND	ND
Benzaldehyde	15	24	29
Phenylacetaldehyde	13	20	18
Octadecanal	ND	ND	55
2,3-pentanedione	7	10	10
3-Hydroxy-2-butanone	20	14	16
1-Hydroxy-2-butanone	5	ND	ND
2,5-Hexanedione	5	ND	ND
2-Pentanone	ND	11	ND

Table 4.20 continues .....

2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	ND	4	7
2-Hydroxy-3,5,5-trimethyl-2-cyclohexenone	ND	42	46
2-Acetoxycyclohexanone	ND	ND	9
3-Methyl-3-nonanone	ND	52	70
3,5,5-trimethyl-2-hexene	18	14	19
1,2,3,4-tetrahydro-6-methyl quinoline	7	ND	ND
1-acetyl-beta-carboline	82	20	10
Total	**1099**	**1957**	**2229**

Hydrolysates 1 and 2 are shrimp hydrolysates at degrees of hydrolysis of 12.2% and 17.1%, respectively.
ND = not detected

Table 4.21 Amounts and number of each class of compounds identified from the thermal reaction of shrimp protein or its hydrolysates[1] with glucose[2].

Compounds	Amounts (ppm)		
	Shrimp protein + glucose	Hydrolysate 1 + glucose	Hydrolysate 2 + glucose
Pyridines	32(3)	ND	ND
Pyrazines	395(6)	599(6)	661(7)
Aldehydes	805(4)	657(4)	861(5)
Furan derivatives	141(7)	133(5)	128(8)
Sulphur-containing compounds	5(1)	13(1)	15(1)
Others	75(17)	92(18)	84(16)
Total	**1453**(38)	**1494**(38)	**1749**(37)

[1]Hydrolysates 1 and 2 are shrimp protein hydrolysates at degrees of hydrolysis of 11.2 and 17.1%, respectively.
[2]The values in parenthesis are number of each class of compounds identified.
ND = Not detected

compounds formed only differed marginally when different hydrolysates with DH values of 11.2 and 17.1%, were used. This could be due to the presence of high levels of free amino acids in both hydrolysates. The content of free amino acids in hydrolysates 1 and 2 were 4.8 and 6.7%, respectively. The gel electrophoresis patterns indicate that rapid and extensive hydrolysis takes place even after 5 min of hydrolysis, and hence a high content of free amino acids and peptides, which are reactants in nonenzymatic browning, would be present in both hydrolysates. Moreover, many volatile compounds were formed in the shrimp protein in about the same concentration as the hydrolysates. The free amino acid content of ground shrimp heads was markedly higher than that of fish (see Tables 4.14 and 4.15 for comparison), by nearly an order of magnitude, thus they may be responsible for generation of high levels of volatile compounds upon reaction with glucose. The total amounts of flavour compounds obtained were 1099, 1957 and 2229 ppm, for shrimp protein, hydrolysate 1 and hydrolysate 2, respectively (Table 4.20). Many of these flavour compounds were formed in heat processing of seal protein hydrolysate without addition of glucose (Hwang *et al.*, 1997). In that study, high levels of aldehydes were particularly noted and indicate that they were mainly formed from lipid degradation. In addition, the presence of endogenous reducing sugars from breakdown of compounds such as nucleotides may also contribute to the formation of the flavour compounds.

Peptides have long been recognized as important compounds in processed flavours. Rizzi (1989) demonstrated that peptides could directly contribute to volatile flavour compounds instead of undergoing hydrolysis of peptide bonds to free amino acids. Experimental results of reactions involving peptides of casein hydrolysates with glucose suggest that peptides contribute directly to volatile compounds formation under thermal reaction conditions, and certain amino acids in the bound form of peptides underwent Strecker degradation to form Strecker aldehydes, which contributed to the formation of corresponding alkylpyrazines in Maillard reaction (Zhang *et al.*, 1992). Ho *et al.* (1992) also demonstrated that peptides play an important role in the generation of pyrazines. They observed that the reactions of glucose with glycine and triglycine generated a larger amount of alkylpyrazines than either di- or tetraglycine.

Besides the sugar-derived carbonyls and furans, the majority of the volatile compounds identified from the three model systems were Strecker aldehydes and their corresponding substituted pyrazines. Although pyrazines are certainly not the only compounds produced by the model systems, however, the conditions selected seem to favour the formation of this group of compounds. A plausible mechanism for the formation of substituted pyrazines may be derived from the model experiments of Chiu *et al.* (1990) and the earlier studies carried out by Shibamato *et al.* (1979). These authors suggested a mechanism for the formation of pyrazines via a 2,5-dihydropyrazine intermediate. The formation of this intermediate was assumed to be from condensation of amino ketones, which were generated during Strecker

degradation. Depending on different amino ketones, the intermediates possess none to three substituents before reacting with aldehydes. The intermediates either underwent dehydrogenation to form pyrazines or reacted with aldehydes to generate pyrazines with corresponding alkyl substitution. These studies were further supported by reactions involving casein protein hydrolysate and glucose (Zhang *et al.*, 1992). The two major Strecker aldehydes identified in this study, involving shrimp protein hydrolysates and glucose, were 3-methylbutanal and phenylacetaldehyde, which were derived from their corresponding free amino acids; leucine and phenylalanine, respectively. These aldehydes have chocolate/floral and honeysuckle favour notes, respectively (Baek and Cadwallader, 1998).

As shown in Table 4.22, the two Strecker aldehydes, namely 3-methylbutanal and phenylacetaldehyde, were present in products of thermal reactions involving both shrimp protein and its hydrolysates with glucose. This indicates that, in the presence of reducing sugars, the Strecker degradation of amino acids proceeded at elevated temperatures in spite of their peptide bondings, which is in agreement with the experimental results of Rizzi (1989) and Zhang *et al.* (1992). In his experiment, Rizzi (1989) heated fructose with different peptides, tripeptides and mixtures of their corresponding amino acids. He found that dipeptides containing valine and leucine produced significant amounts of Strecker aldehydes, 2-methylpropanal and 3-methylbutanal, despite the blocked amino or carboxyl groups. Similarly, Zhang *et al.* (1992) reported the presence of 3 Strecker aldehydes which were derived from their corresponding free amino acids upon reaction of casein hydrolysate and its peptides

Table 4.22 Aldehyde compounds identified in reaction of shrimp protein or its hydrolysates[1] with glucose.

Compound	Concentration (ppm)		
	Shrimp protein + glucose	Hydrolysate 1 + glucose	Hydrolysate 2 + glucose
3-Methylbutanal	107	516	639
2-Methylbutanal	ND	97	120
Hexanal	6	ND	ND
Benzaldehyde	15	24	29
Phenylacetaldehyde	13	20	18
Octadecanal	ND	ND	55
Total	**141**	**657**	**861**

[1]Hydrolysates 1 and 2 are shrimp protein hydrolysates at degrees of hydrolysis of 11.2 and 17.1%, respectively.
ND = Not detected

with glucose at 180 °C. These results suggest that additional steps, such as hydrolysis of peptide bonds, might be involved in the Strecker aldehyde formation from peptides compared to that from free amino acids. The compound, 3-methylbutanal was the most abundant volatile compound in the hydrolysates. The aldehydes were formed either from lipid degradation or from Strecker degradation of amino acids. The flavour notes of the aldehydes are noteworthy because of the high concentrations generated in these reactions. Benzaldehyde has a pleasant almond, nutty and fruity flavour while hexanal has a green, grassy, or apple-like flavour (Vejaphan *et al.*, 1988; Hsieh *et al.*, 1989)

Pyrazines form a major component of flavour volatiles in heat processed reactions (Shibamoto *et al.*, 1979). More than 100 pyrazines have been identified in various food products (Maga, 1983). In this study, many pyrazines were formed more abundantly from the hydrolysates than from the control. For example, the concentration of 3-ethyl-2,5-dimethylpyrazine was 5.5 and 6.5 times higher in hydrolysate 1 (DH=12.2%) and hydrolysate 2 (DH=17.1%), respectively, as compared with that of the control (Table 4.23). The most abundant pyrazine was 2,5-dimethylpyrazine and this compound contributed 50.9, 55.1 and 50.2% to the total amount of pyrazines identified in reactions involving glucose with shrimp protein, hydrolysate 1 and hydrolysate 2, respectively. The next most abundant pyrazine was methylpyrazine followed by trimethylpyrazine. Pyrazines are known to have roasted flavours and methylpyrazines contribute to the roasted aroma of clam more than other

Table 4.23 Pyrazines identified in reaction of shrimp protein or its hydrolysates[1] with glucose.

Compound	Concentration (ppm)		
	Shrimp protein + glucose	Hydrolysate 1 + glucose	Hydrolysate 2 + glucose
Pyrazine	17	trace	43
Methylpyrazine	90	118	134
2,5-dimethylpyrazine	201	330	332
2,3-dimethylpyrazine	13	16	16
Trimethylpyrazine	72	124	123
3-ethyl-2,5-dimethylpyrazine	2	11	13
Total	**395**	**599**	**661**

[1]Hydrolysates 1 and 2 are shrimp protein hydrolysates at degree of hydrolysis 11.2 and 17.1%, respectively.

volatile components (Kubota *et al.*, 1991). Pyrazines identified in shellfish are mainly alkylpyrazines (Pan and Kuo, 1994). The alkylpyrazines generally possess roasted, nut-like flavour notes (Maga, 1983).

Other nitrogen-containing compounds, such as pyridines and cyclic dipeptides, were found in smaller amounts than pyrazines. Furan-derived compounds were also found (Table 4.24). These compounds generally possess sweet or caramel-like flavour notes. Based on the flavour notes of volatiles generated from this reaction, shrimp protein hydrolysates may potentially be used as flavour precursors to impart flavour to some processed surimi-based foods and other products.

4.13 Yield, total phenolics and carotenoids contents of shrimp ethanolic extracts

Among the potential solvents considered for extraction of crude antioxidant components in shrimp, acetone, chloroform, diethyl ether and ethanol were examined in this study. Preliminary results of antioxidant activity of extracts indicated that ethanol was the most effective extraction medium, and hence was the solvent of choice. The yield of crude ethanolic extract from different parts of shrimp is given in Figure 4.42. The flesh gave the highest crude ethanolic extract of 7.8%, on a wet basis. The heads, whole shrimp and shell extracts were next on the basis of increasing yield, respectively. The crude extract may, however, contain other components, which may not have antioxidant activity. On a dry basis, the shells afforded the highest yield of crude extract.

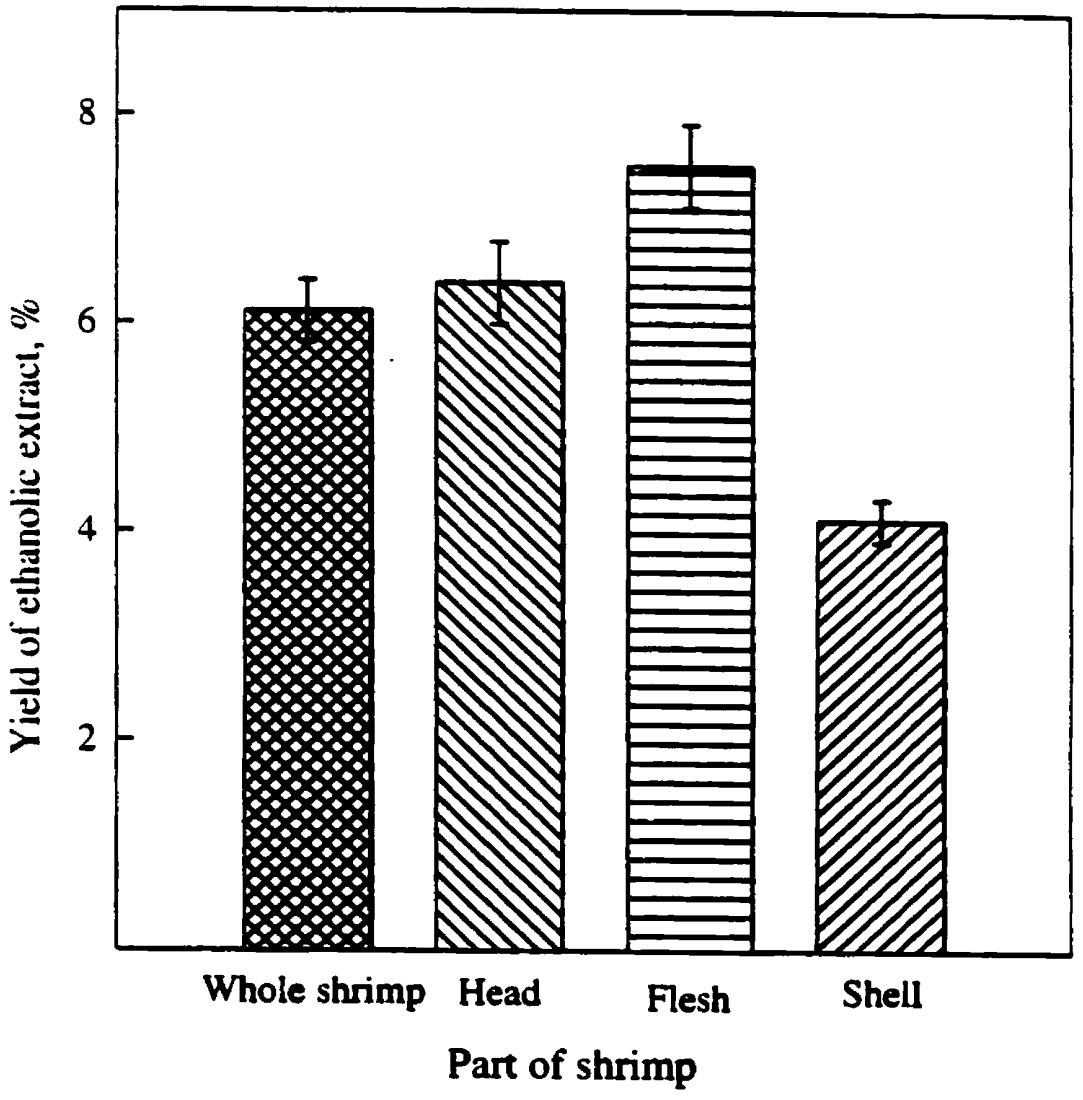
Table 4.24 Furan-derived compounds identified in reaction of shrimp protein or its hydrolysates[1] with glucose.

Compound	Concentration (ppm)		
	Shrimp protein + glucose	Hydrolysate 1 + glucose	Hydrolysate 2 + glucose
Dihydro-2-methyl-3-furanone	7	14	17
2-furfural	34	44	45
2-furanmethanol	4	ND	ND
2-acetylfuran	7	ND	ND
5-methyl-2-furfural	trace	10	10
5-(hydroxymethyl) 2-furfural	ND	10	4
Total	**52**	**78**	**76**

[1] Hydrolysates 1 and 2 are shrimp protein hydrolysates at degrees of hydrolysis of 11.2 and 17.1%, respectively.

ND = Not detected

Figure 4.42 Yield of crude ethanolic extract of shrimp body parts.


8
6
4
2
Yield of ethanolic extract, %
Whole shrimp
Head
Flesh
Shell
Part of shrimp

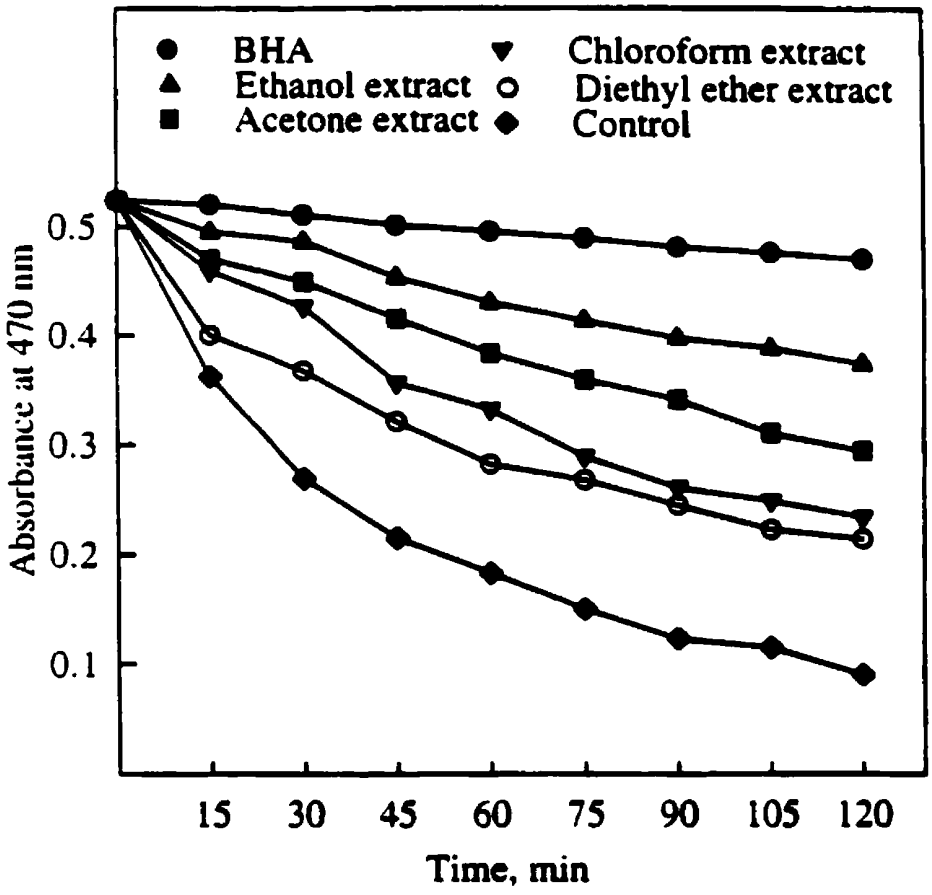
The total phenolics content of different shrimp parts indicates that shells also constitute a rich source of phenolic compounds in shrimp. Phenolics amounted to 0.28, 0.21, 0.19 and 0.15 mg/g sample, expressed as gallic acid equivalents for shells, whole shrimp, flesh and heads, respectively. The enzyme phenoloxidase, which catalyzes o-hydroxylation of monophenols and dehydrogenation of o-diphenols is present in shrimp and lobster (Savagaon and Screenivasan, 1978). This enzyme is responsible for melanosis (a surface decoloration caused by enzymatic formation of precursor compounds which polymerize to from insoluble pigments) in crustaceans. The presence of this enzyme in shrimp is a likely indication of the presence of phenolic compounds which are substrates for the enzyme. The distribution of phenolic compounds in different body parts of shrimp may be due to the specific functions of these compounds in the parts concerned. Phenolic compounds play an important role in tissue regeneration and healing by their interaction with proteins via the functional groups of amino acids. The presence of phenolic compounds in shrimp was also confirmed by the appearance of a brownish-yellow colouration when thin layer chromatographic plates of the extract were sprayed with a ferric chloride reagent (Reio, 1958). Phenolic compounds in shrimp may exist as intermediates of some metabolic pathways or breakdown products of other compounds or as end products of certain metabolic pathways, formed mainly in tissue as a protection against oxidative damage. They may also have roles in other physiological functions which are yet to be determined.

Carotenoids are present in some crustaceans such shrimp, crab and lobster as well as salmonid fish such as salmon, charr and rainbow trout. The total carotenoid content of the different parts of shrimp indicated that the highest amount was present in the shells, at approximately 18 μg/g sample, and those of whole shrimp, heads and flesh were 16.4, 11.1 and 7.2 μg/g sample. Apart from their desirable characteristic colour, carotenoids may also possess antioxidant activity (King *et al.*, 1993). A possible synergism between phenolic compounds and carotenoids may be responsible for the observed antioxidant activity of shrimp (Boldyrev *et al.*, 1988; Chen *et al.*, 1997). The phenolic compounds or the carotenoids may also exert their effects independent of each other.

4.14 Antioxidant activity of the crude shrimp extract

The effect of antioxidative components extracted into different solvents, as examined in a β-carotene/linoleate model system, is summarised in Figure 4.43. Amongst the solvents with varying degrees of polarity used in the extraction, absolute ethanol was most effective. In general, the degree of effectiveness of the extraction process, based on the antioxidant activity of the crude extracts, was in the order of ethanol > acetone > chloroform > diethyl ether. The effective extraction of the antioxidant components in ethanol is likely due to the polar nature of these compounds. The presence of phenolic compounds in the extract gives more impetus to the possible role of these compounds as major antioxidant components of shrimp.

Figure 4.43 Antioxidant activity of crude extracts of shrimp heads as assessed in a β-carotene-linoleate model system.


BHA
Ethanol extract
Acetone extract
Chloroform extract
Diethyl ether extract
Control
Absorbance at 470 nm
0.5
0.4
0.3
0.2
0.1
15
30
45
60
75
90
105
120
Time, min

The presence of hydroxylated compounds in shrimp has been reported (Pasquel and Babbitt, 1991; Li *et al.*, 1996). Although other solvents such as acetone, chloroform and diethyl ether were less effective than ethanol in the extraction of the antioxidant compounds in shrimp, all solvents used were able to extract these compounds to varying degrees. The antioxidant activity of the extracts of shrimp, using the different organic solvents, in comparison with BHA, shows that extracts obtained in four solvents possessed antioxidant activity, as indicated by delayed bleaching of β-carotene in an emulsion system. Based on the results of the antioxidant activity of these extracts, ethanol was most effective as an extraction medium and hence used for subsequent extraction experiments related to studies on antioxidant components in shrimp.

4.14.1 Isolation of antioxidative compounds of shrimp using chromatographic techniques

The crude extract of shrimp obtained after extraction with 95% (v/v) ethanol was separated using a silica gel column. No single solvent system was effective in separating all components of the crude extract, hence two solvent systems with different polarities were used. Solvent I (n-hexane/acetone, 3:1, v/v) was used to elute the less polar components which were collected in 25 mL fractions, six fractions (fractions 1-6) were collected. The more polar components were eluted with n-butanol-water-acetic acid (3:1:1, v/v/v). Five fractions (fractions 7-11) were collected using this solvent. Amarowicz *et al.* (1997) employed two solvent systems to separate

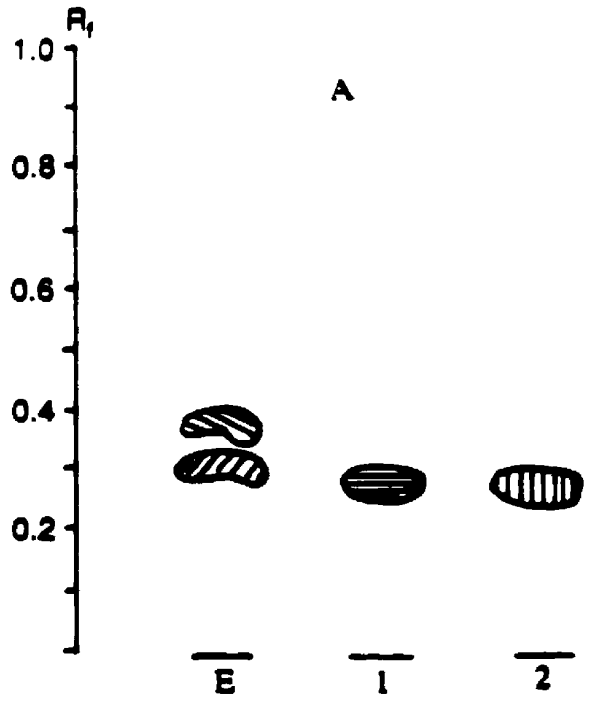
flaxseed extracts using silica gel column chromatography. TLC separation of different fractions indicated the presence of phenolic compounds which became brownish-yellow upon spraying of the plates with a ferric chloride reagent.

Fractions 3-6 separated by solvent I and fractions 7-11 separated by solvent II were pooled together and used for analytical TLC. Solvent I was not sufficiently polar for developing of compounds from Fractions 7-11. Bands 1 and 2 (obtained from fractions 3-6) were less polar than bands 3 and 4 (obtained from fractions 7-11). Thus, the R_f values of compounds 1 and 2 were 0.30 and 0.29, respectively, when solvent I was used for developing the plates. However, a second chromatogram was obtained when solvent II was used and thus compounds 3 and 4 having R_f values of 0.54 and 0.34, respectively, were obtained (Figure 4.44). When another sets of plates were sprayed with a β-carotene-linoleate solution in order to evaluate the antioxidant activity of the spots, all separated components delayed the bleaching of β-carotene, thus indicating that they were antioxidative in nature.

4.14.2 Isolation of individual compounds by reversed phase HPLC

The semi-preparative HPLC chromatograms of the four compounds obtained by preparative TLC are given in Figure 4.45. When concentrations of 20 to 90% (v/v) methanol were used, the various compounds were not eluted from the column, but only 100% methanol was effective as the elution solvent. The retention times of these peaks were 13 min (compound 1), 47 min (compound 2), 21 min (compound 3) and 16 min (compound 4). However, the retention times of the compounds when

Figure 4.44 TLC plates of compounds isolated from ethanolic shrimp extract. Plates A and B were developed using n-hexane/acetone and n-butanol/water/acetic acid, respectively.


R_f
1.0
0.8
0.6
0.4
0.2
A
E
1
2

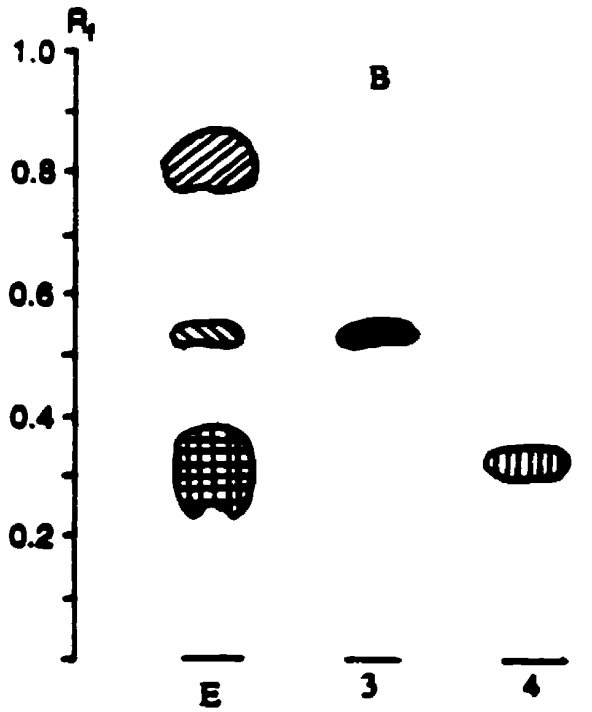
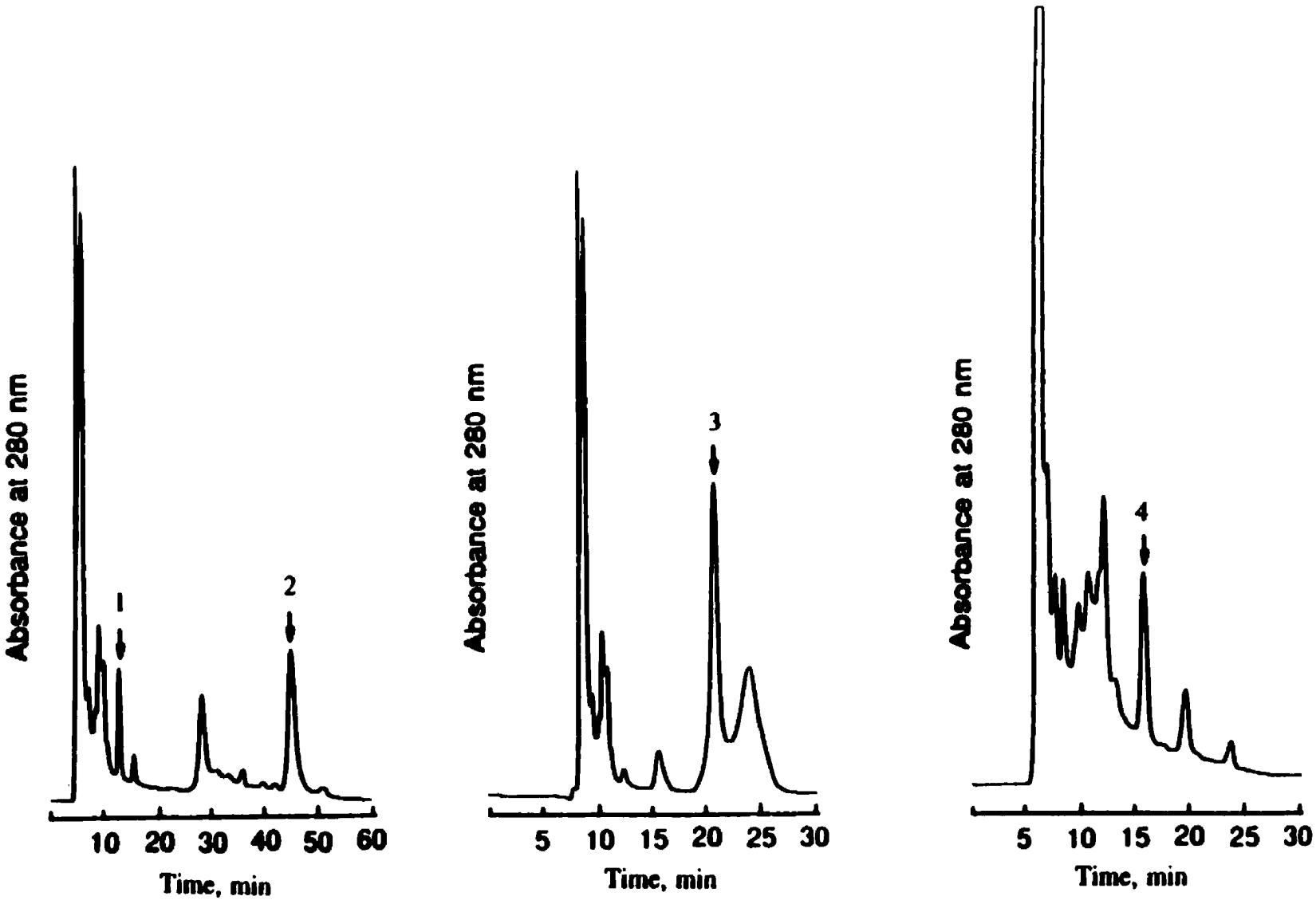

R_f
1.0
0.8
0.6
0.4
0.2
B
E
3
4

Figure 4.45 Semi-preparative reversed-phase HPLC chromatograms of shrimp extract.


Absorbance at 280 nm
1
2
10 20 30 40 50 60
Time, min
Absorbance at 280 nm
3
5 10 15 20 25 30
Time, min
Absorbance at 280 nm
4
5 10 15 20 25 30
Time, min

analytical reversed-phase HPLC was employed were 4 min (compound 1), 14 min (compound 2), 8 min (compound 3) and 3 min (compound 4) [Figure 4.46]. The purity of the isolated compounds was confirmed by analytical TLC. The isolated compounds dissolved in methanol were subjected to UV/visible spectroscopy. The UV/visible spectra of these compounds are given in Figure 4.47. The absorption maxima of compounds 1, 3 and 4 were at 270, 280 and 274 nm, respectively. However compound 4 had multi-peak absorptions at 262, 272, 282 and 294 nm. The reasons for this type of absorption spectrum for compound 4 remains unknown; the possibility of the presence of impurities has not been excluded.

4.14.3 Structural elucidation of antioxidative compounds of shrimp using electrospray mass spectrometry (ESMS)

The negative electrospray mass spectrum of compound 2, $C_{18}H_{22}N_2O$ (MW 282) was recorded with a cone-voltage of 75 volts and shown in Figure 4.48. The fragmentation patterns of the deprotonated molecule $[M-H]^-$ at m/z 281 have been tentatively rationalized and summarized in Figure 4.49, as follows: The deprotonated molecule $[M-H]^-$ at m/z 281 may lose a molecule of acetylene (C_2H_2; 26 amu) to afford the $[M-H-CH=CH]^-$ deprotonated fragmented ion at m/z 255. The deprotonated molecule $[M-H]^-$ may also lose a molecule of methylene amine ($CH_2=NH$) to afford the deprotonated fragment-ion $[M-H-CH_2NH_2]^-$ at m/z 253. The deprotonated fragment ion $[M-H-CH=CH]^-$ at m/z 255 loses consecutively, a molecule of ketene

Figure 4.46 Analytical reversed-phase HPLC chromatograms of shrimp extract.

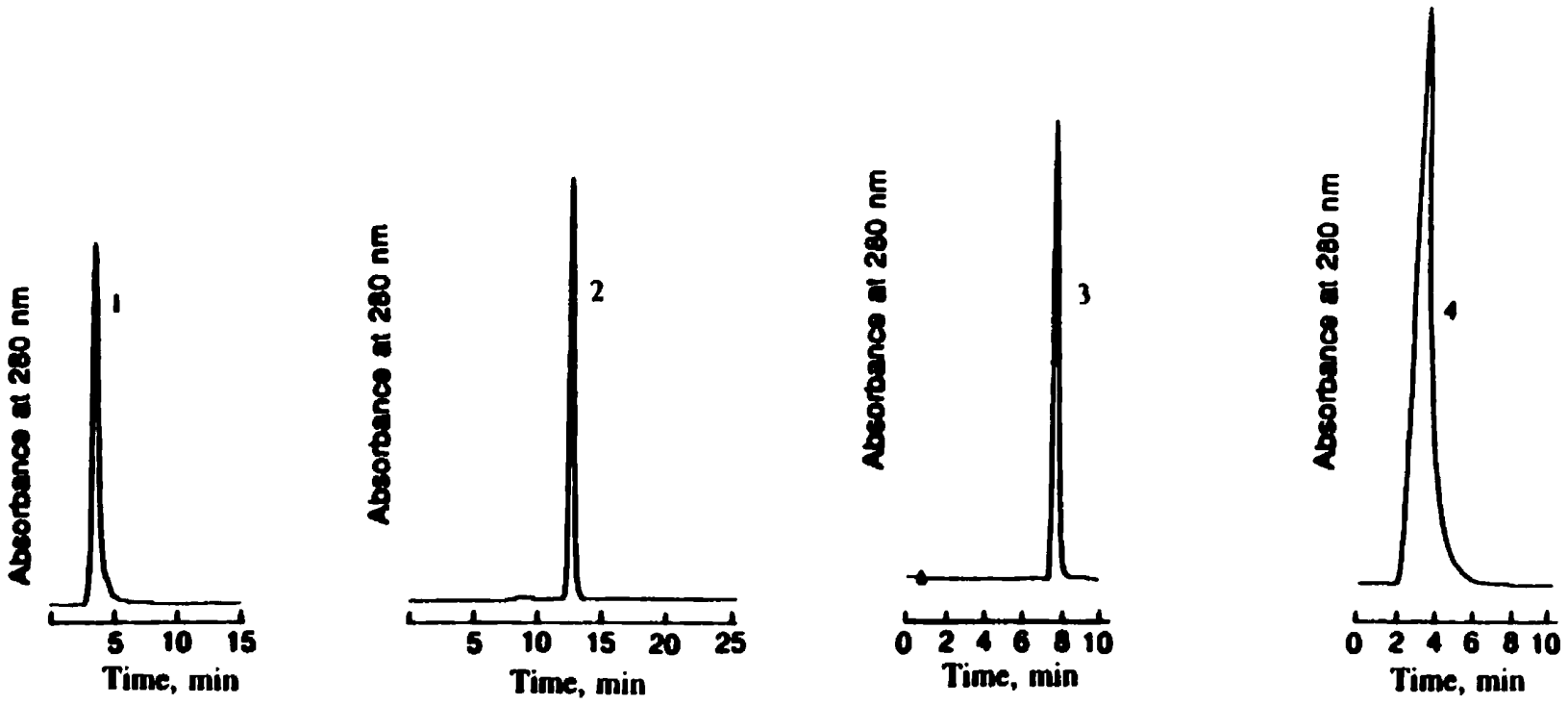
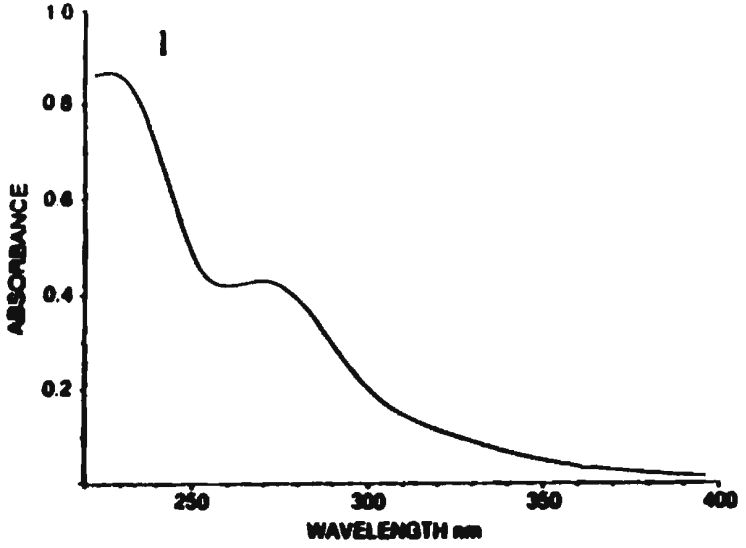
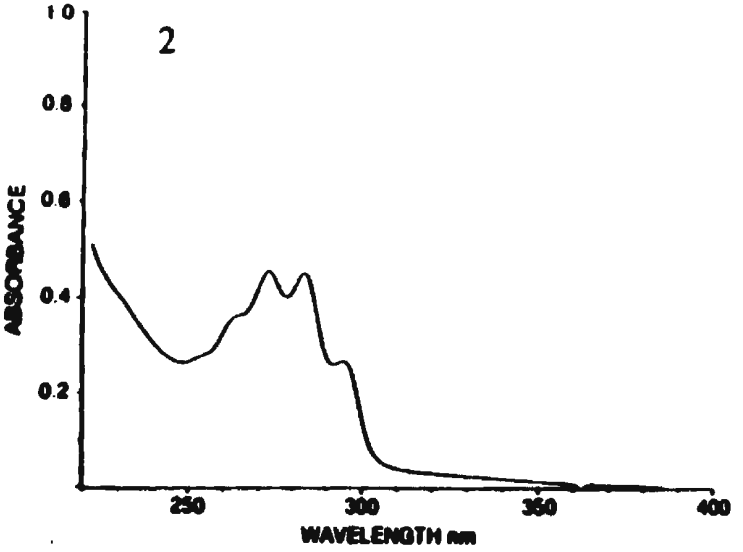
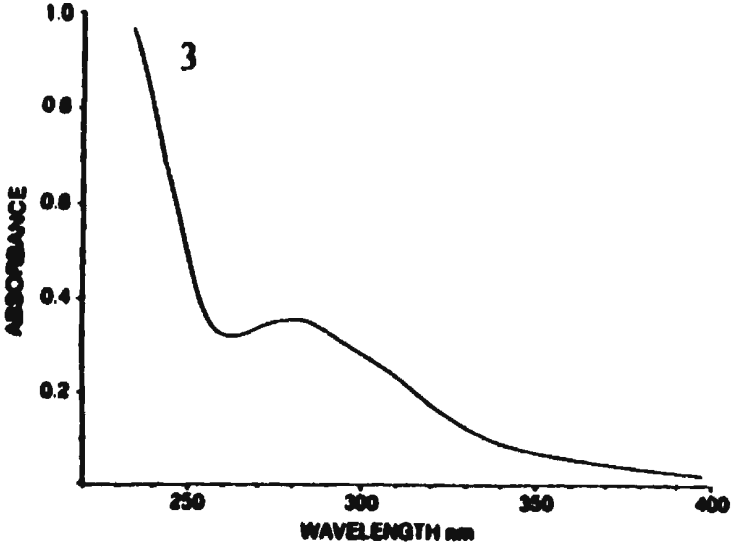

Absorbance at 280 nm
1
5 10 15
Time, min
Absorbance at 280 nm
2
5 10 15 20 25
Time, min
Absorbance at 280 nm
3
0 2 4 6 8 10
Time, min
Absorbance at 280 nm
4
0 2 4 6 8 10
Time, min

Figure 4.47 UV-visible spectra of isolated Compounds 1-4 of shrimp.


1
1.0
0.8
0.6
0.4
0.2
ABSORBANCE
250
300
350
400
WAVELENGTH nm


2
1.0
0.8
0.6
0.4
0.2
ABSORBANCE
250
300
350
400
WAVELENGTH nm


3
1.0
0.8
0.6
0.4
0.2
ABSORBANCE
250
300
350
400
WAVELENGTH nm

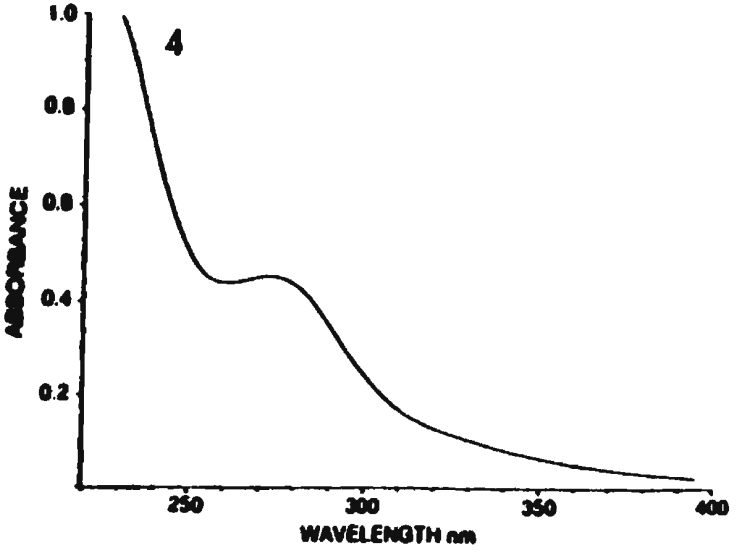

4
1.0
0.8
0.6
0.4
0.2
ABSORBANCE
250
300
350
400
WAVELENGTH nm

Figure 4.48 Negative electrospray mass spectrum of compound 2, m/z 282.

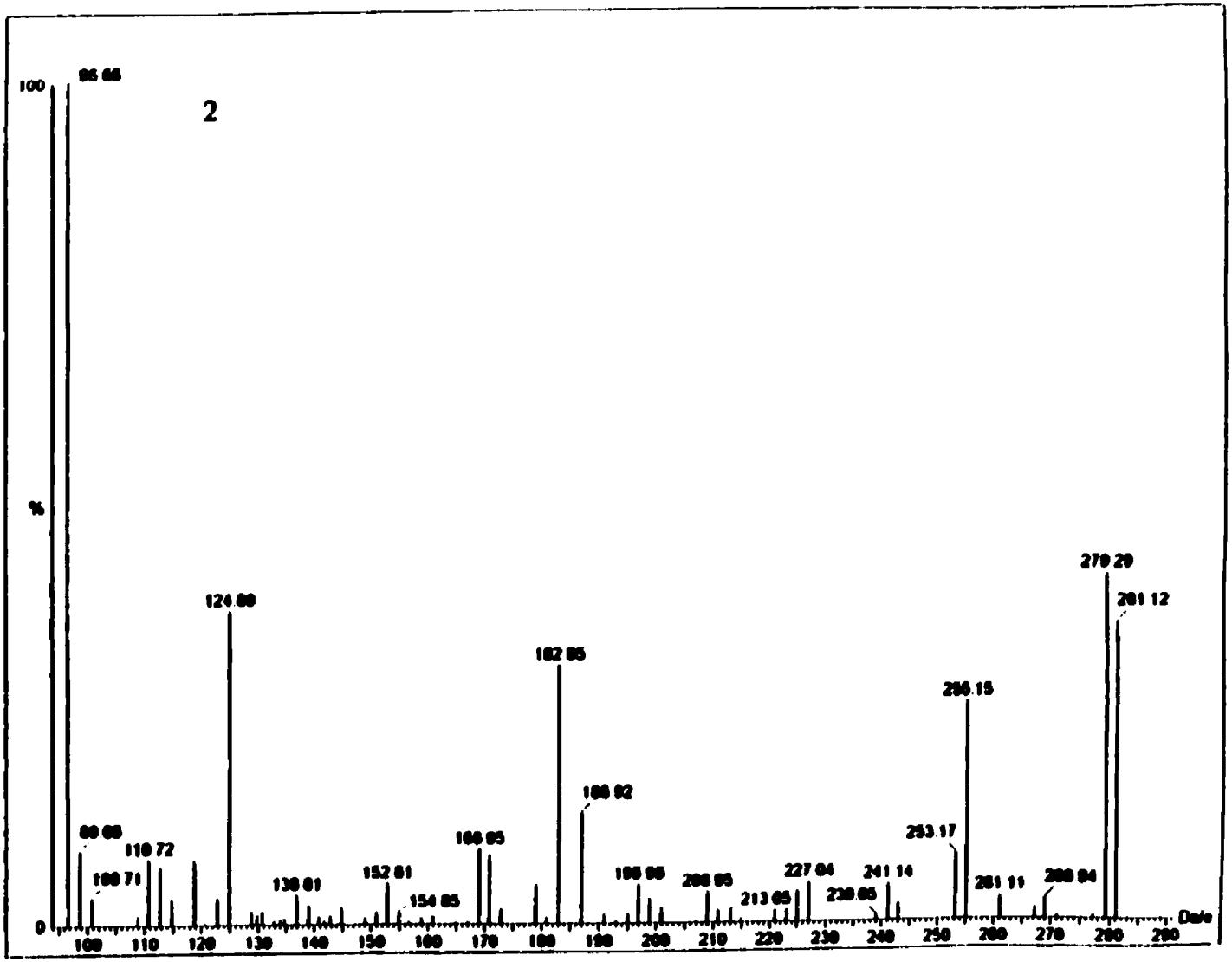
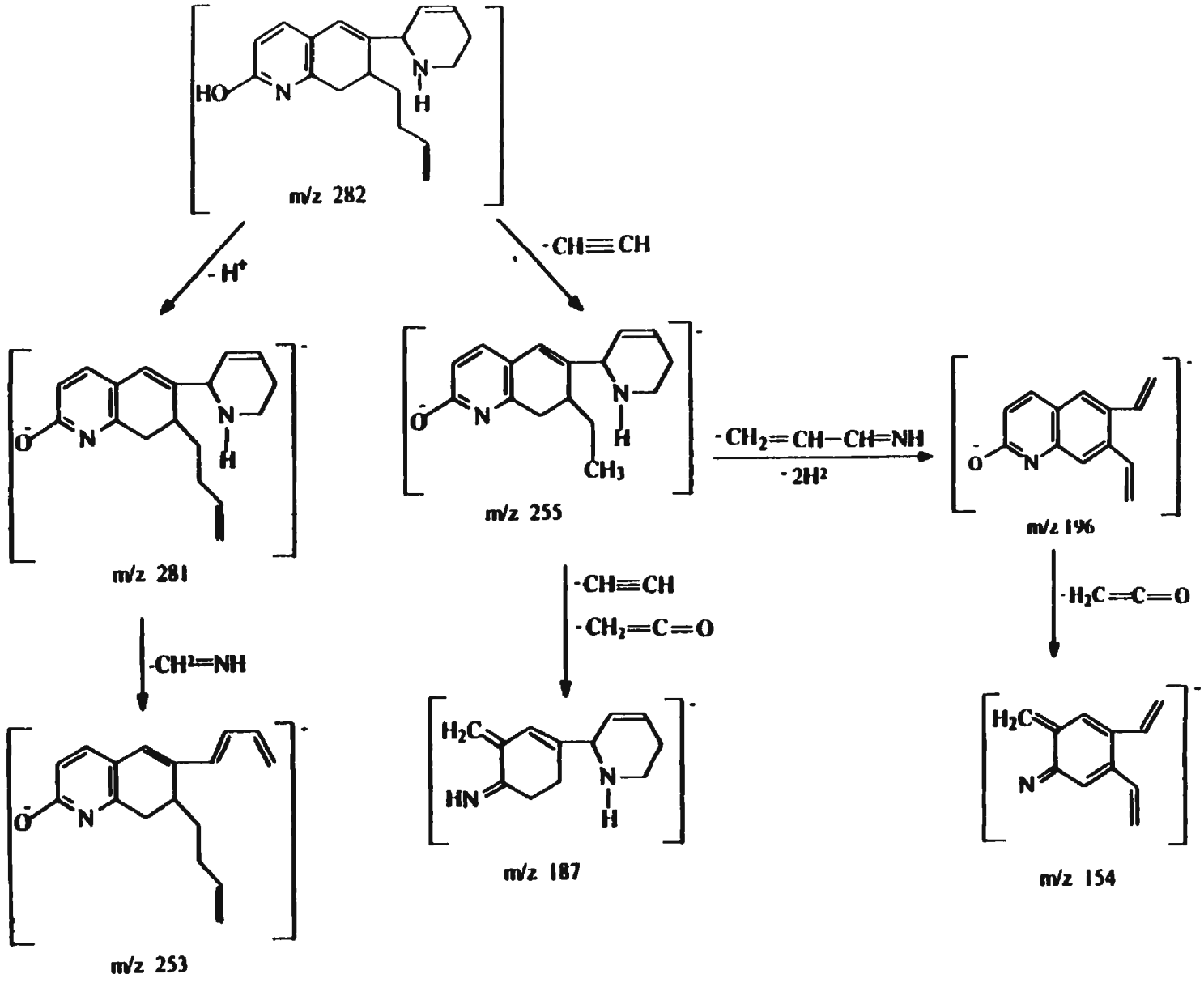

2
100
%
0
95.66
124.88
182.85
279.29
281.12
256.15
186.92
253.17
110.72
88.68
100.71
138.81
152.81
154.85
168.95
186.86
208.85
213.85
227.84
230.85
241.14
281.11
288.84
Daltons
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240
250
260
270
280
290

Figure 4.49 Proposed scheme for fragmentation of compound 2, m/z 282, identified as 7-(3-butenyl)-2-hydroxy-6-(1,2,5,6-tetrahydropyridin-2-yl)-7,8-dihydroquinoline.


m/z 282
-H•
-CH≡CH
m/z 281
m/z 255
-CH₂=CH-CH=NH
-2H²
m/z 196
-CH²=NH
-CH≡CH
-CH₂=C=O
-H₂C=C=O
m/z 253
m/z 187
m/z 154

(CH_2CO; 42 amu) and a molecule of acetylene (C_2H_2; 26 amu) to afford the deprotonated fragment-ion $[M\text{-}H\text{-}C_2H_2\text{-}CH_2CO\text{-}C_2H_2]^-$ or $[M\text{-}H\text{-}2(C_2H_2)\text{-}CH_2CO]^-$ at m/z 187. The deprotonated fragment-ion at m/z 255 can also lose simultaneously a molecule of propyleneamine (CH_2=CH-CH=NH) and 2 molecules of hydrogen (or vice versa) to afford the deprotonated fragment-ion $[M\text{-}H\text{-}C_2H_2\text{-}CH_2\text{=}CH\text{-}CH\text{=}NH\text{-}2(H_2)]^-$ at m/z 196. This latter deprotonated fragment-ion undergoes a retro-Diels Alder rearrangements and its further aromatization by loss of 2 hydrogen atoms affords the deprotonated fragment ion at m/z 154. Thus, compound 2 was identified as 7-(3-butenyl)-2-hydroxy-6-(1,2,5,6-tetrahydropyridin-2-yl)-7,8-dihydroquinoline.

The negative electrospray mass spectrum of compound 4, $C_{18}H_{20}N_2O$ (MW. 280), was recorded with a cone voltage of 75 volts, is presented in Figure 4.50. The fragmentation routes of the deprotonated molecule [M-H] at m/z 279 have been tentatively rationalized and are summarized in Figure 4.51, as follows: The deprotonated molecule $[M\text{-}H]^-$ at m/z 279 loses simultaneously, by a concerted mechanism, a molecule of CH_2=CH-OH (44 amu) and by a retro-Diels Alder rearrangement and a molecule of CH_2=CH-C≡CH (52 amu) to afford the deprotonated fragment-ion $[M\text{-}H\text{-}C_2H_4O\text{-}C_2H_4O\text{-}C_4H_4]^-$ at m/z 183. This latter deprotonated fragment-ion looses consecutively the side chain portion, that is, a molecule of CH_2=CH-CH=CH_2, 154 amu and 2 molecules of hydrogen to afford the deprotonated fragment-ion at m/z 126 which ultimately looses a molecule of HCN to afford the

Figure 4.50 Negative electrospray mass spectrum of compound 4, m/z 280.

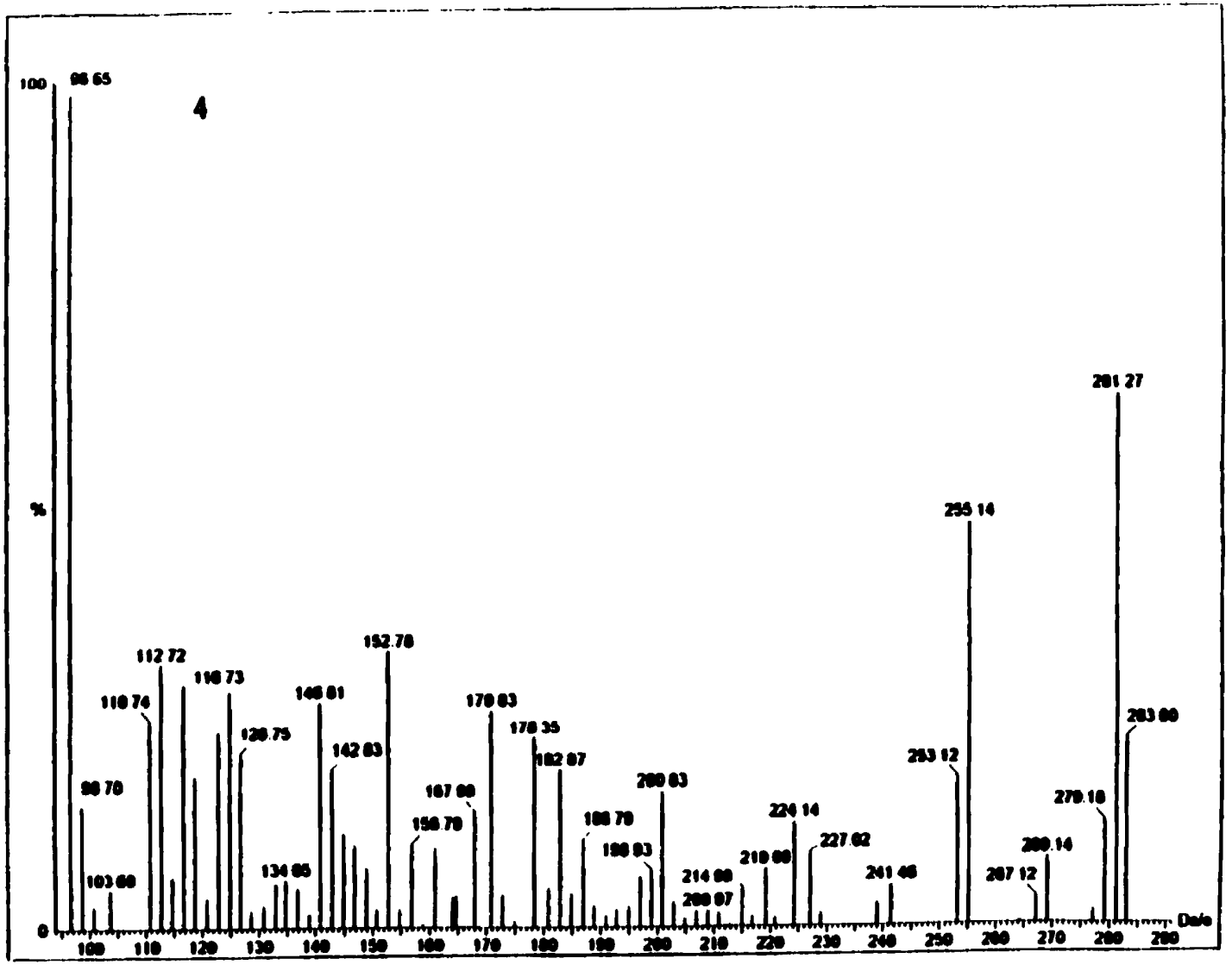
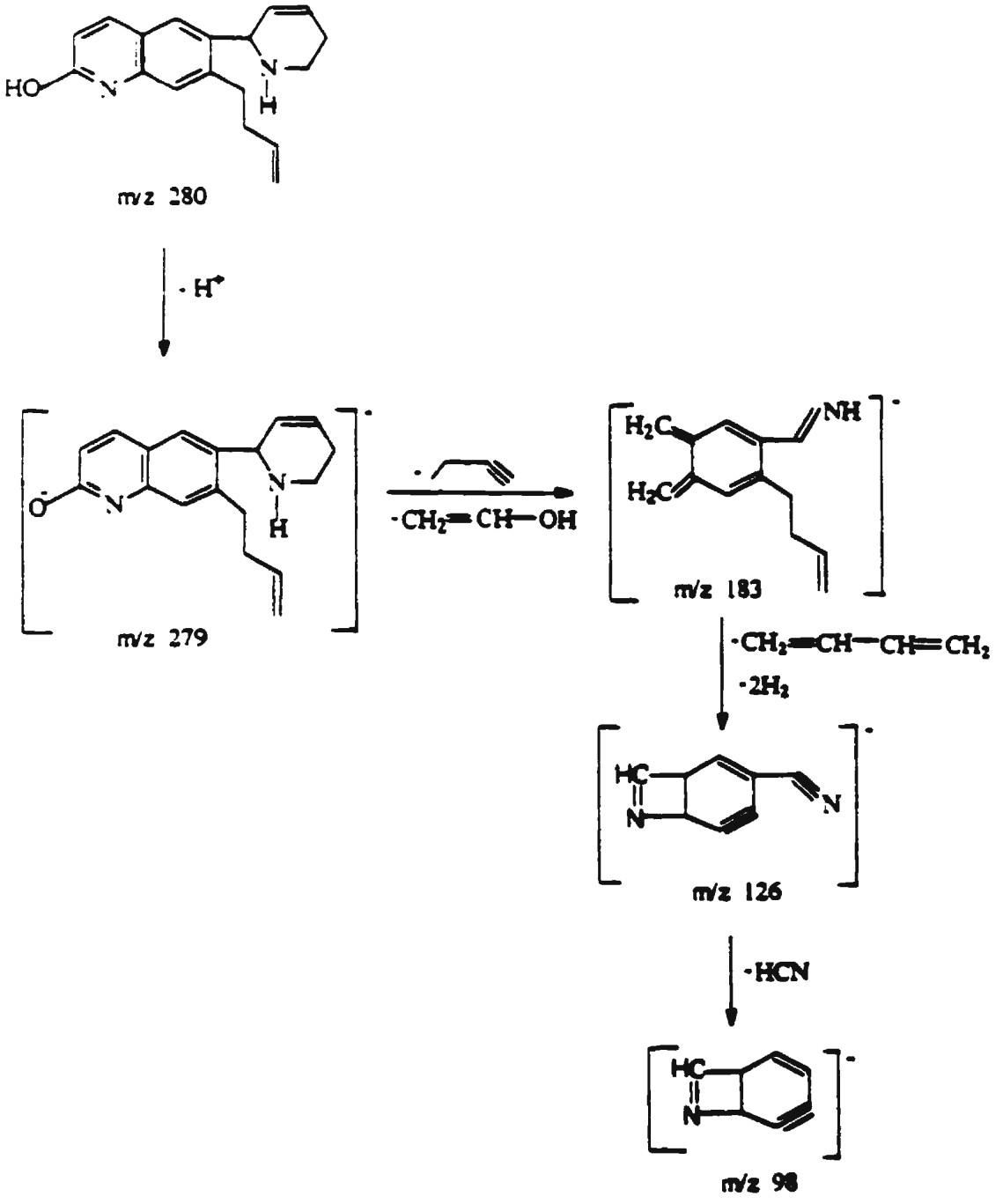

4
100
%
0
98.65
112.72
116.73
110.76
120.75
146.81
142.83
152.78
98.70
103.69
134.85
156.79
167.80
170.83
178.35
182.87
188.70
200.83
198.83
214.80
208.87
219.80
224.14
227.62
241.46
253.12
255.14
279.18
281.27
283.80
287.12
289.14
Da/e
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240
250
260
270
280
290

Figure 4.51 Proposed scheme for fragmentation of compound 4, m/z 280, identified as 7-(3-butenyl)-2-hydroxy-6-(1,2,5,6-tetrahydropyridin-2-yl) quinoline.


HO
N
H
m/z 280
· H·
O
N
H
m/z 279
·CH₂═CH—OH
H₂C
NH
H₂C
m/z 183
·CH₂═CH—CH═CH₂
·2H₂
HC
N
N
m/z 126
·HCN
HC
N
m/z 98

deprotonated fragment ion at m/z 98. Thus, compound 4 was identified as 7-(3-butenyl)-2-hydroxy-6-(1,2,5,6-tetrahydropyridin-2-yl) quinoline.

Identification of compounds 1 and 3 whose electrospray mass spectra (negative ion mode) are given in Figures 4.52 and 4.53, respectively, indicated that they were isomers of compounds 2 and 4, respectively. Indeed, the ESMS spectra for compounds 1 and 3 were identical to those corresponding ESMS for compounds 2 and 4. This indicates the existence of possible geometrical isomers. These isomers may differ in the steric geometry around the double bonds and orientation of groups present in the molecules. However, due to difficulties experienced in isolation of even small amounts of purified samples, other spectroscopic analyses could not be performed to further confirm these chemical structures.

Although a wealth of structural information is obtained from these ESMS spectra, it is very difficult to establish the genealogy of the fragment ions produced. In particular, the spectra were complicated by ions arising from a series of multiple fragmentations which yielded intermediate ions of very low abundance. We have not attempted to rationalize the different fragmentation routes of these antioxidant compounds by obtaining precursor ion spectra of various intermediate ions. Assignment of fragment ion structures was based mainly on the tentative interpretation of selected precursor ions. The elimination sequences of the neutral molecules from the various fragment ions are numerous and their verification would require the synthesis of suitable molecules containing stable ^{13}C and ^{18}O isotopes.

Figure 4.52 Negative electrospray mass spectrum of compound 1.

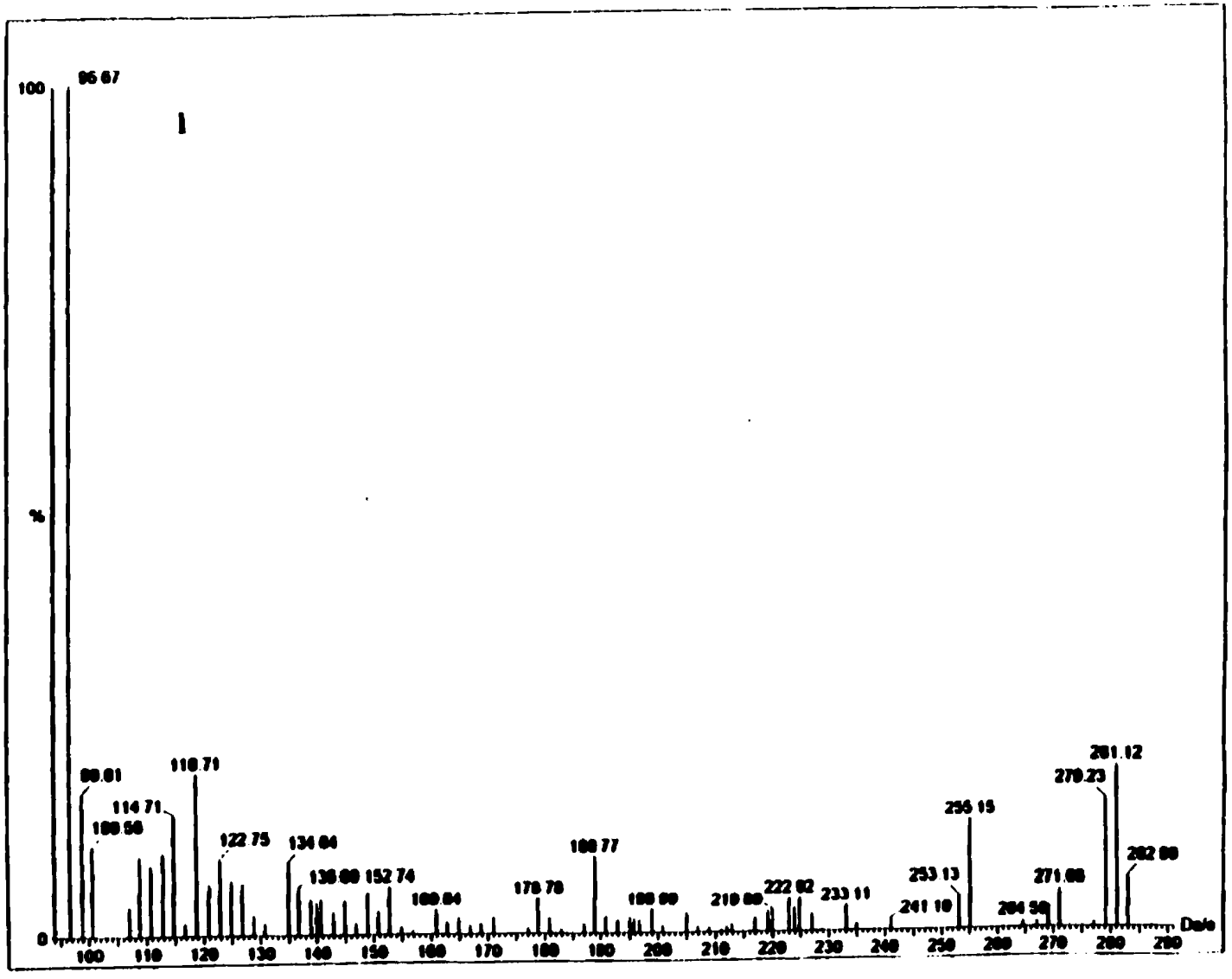
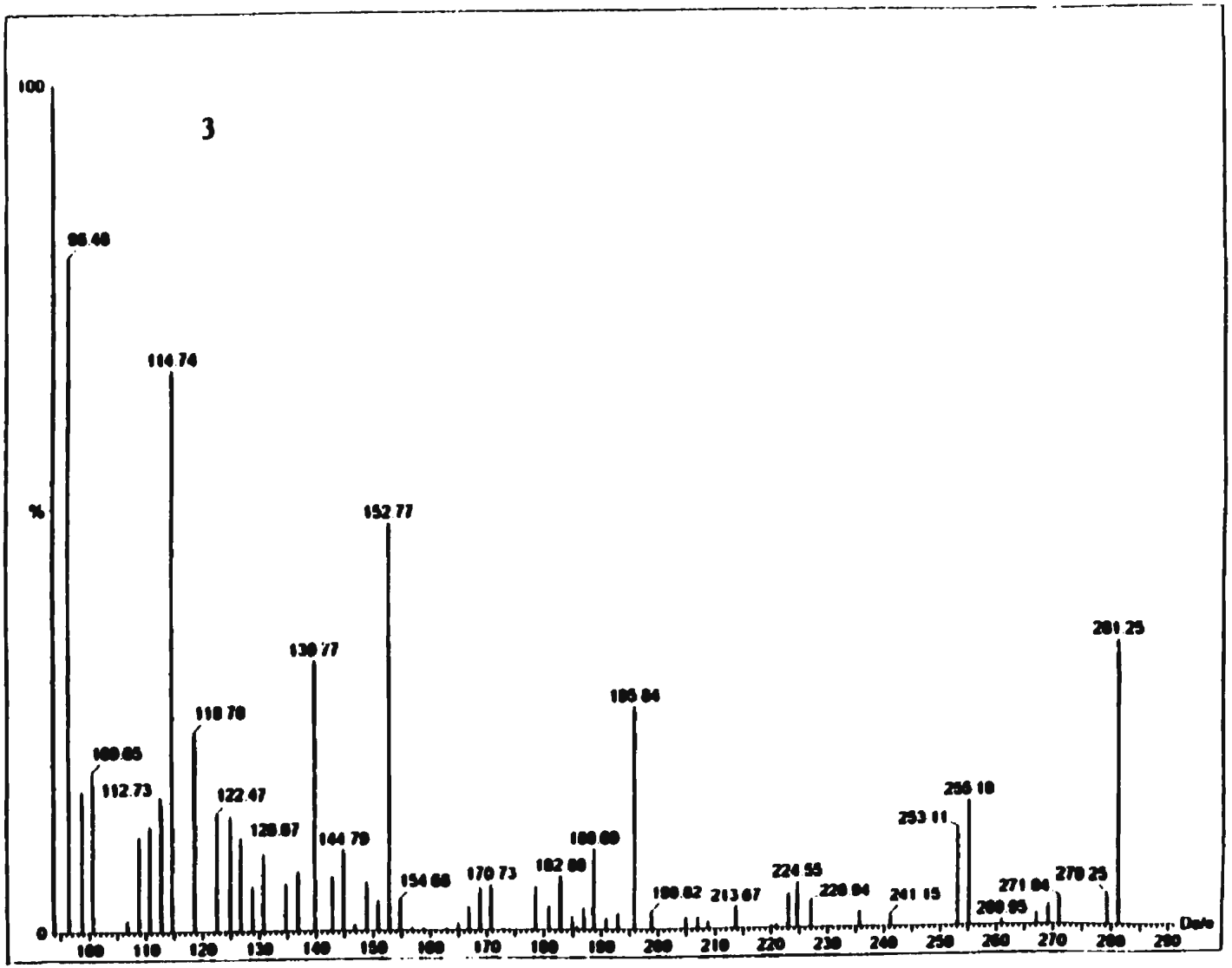

100
%
0
96.67
89.81
114.71
116.71
122.75
134.64
152.74
178.78
188.77
222.82
233.11
253.13
255.15
271.68
279.23
281.12
282.89
Daltons

Figure 4.53 Negative electrospray mass spectrum of compound 3.


3
100
%
0
96.48
116.74
152.77
139.77
118.78
186.84
281.25
109.65
112.73
122.47
128.87
144.79
154.68
170.73
182.80
189.69
199.82
213.67
224.55
228.94
241.15
253.11
256.10
271.84
278.25
288.95
Daltons

CONCLUSIONS AND MAJOR CONTRIBUTIONS OF THIS RESEARCH

The preparation of protein dispersions from capelin and shark meats was carried out, thus affording highly viscous and thermostable products. However, almost complete solubilization of shark protein was achieved only after acidification, which afforded a low viscous dispersion. The nutritional value of the proteins, with respect to the amino acid composition and calculated protein efficiency ratio was not compromised in the preparation of products. The protein dispersions could find suitable uses in a number of fabricated food products.

Enzymatic hydrolysis of shrimp heads and lumpfish protein was accomplished under optimum conditions, using Alcalase, by employing response surface methodology (RSM). The degree of hydrolysis and nitrogen recovery obtained from predicted and experimental conditions were close and afforded hydrolysates which were highly soluble in water (over 94%) in the pH range 2-10. Other unique functional properties with respect to emulsifying properties, fat and moisture adsorptions, whippability and foam stability, were noted in the protein hydrolysates. Some of these functional properties of the hydrolysates which are also exhibited by fat may lead to the use of protein hydrolysates as fat replacers in certain food systems. The presence of protein hydrolysates at varying concentrations (0-3%, w/w) enhanced the yield and lowered the drip loss of meat upon heat processing. This influence of shrimp protein hydrolysate was comparable to that of selected phosphates (sodium hexametaphosphate) and hence shrimp protein hydrolysate may be used as an

alternative to phosphates commonly used in food. Furthermore, antioxidant activity of protein hydrolysates was demonstrated in a β-carotene/linoleate and meat model systems. Sephadex G-15 column separation of shrimp protein hydrolysate resulted in 5 fractions which were antioxidative. Reversed-phase high performance liquid chromatography of column chromatographic fractions afforded separation of multipeak eluates. The protein hydrolysates would confer antioxidant effect in food formulations in addition to their specific functional properties.

The high level of free amino acids and peptides in protein hydrolysates was exploited in the thermal generation of aroma in the presence of glucose. Over 30 flavour compounds were identified in a Maillard-type reaction involving shrimp protein or its hydrolysates and glucose. These are known to have different flavour notes and hence shrimp protein hydrolysates may be used as flavouring components in selected heat-processed foods where their specific aroma might be desirable.

The unique aspects of functional, antioxidative and other properties of protein hydrolysates are strong arguments for the use of marine-based hydrolysates in food applications. The hydrolysates may also be incorporated into many food systems such as low pH beverages and diets that are suitable for geriatrics, high-energy supplements, weight-control diets, hypoallergenic infant formulas and therapeutic or enteric diets as well as nonfood applications such as nitrogen source for fermentation media formulations and bait in the fishing industry.

The presence of antioxidant compounds in shrimp was established. These compounds were isolated and characterized using TLC, HPLC and electrospray mass spectrometry. The compounds are 3-membered ring phenolics and were identified as 7-(3-butenyl)-2-hydroxy-6-(1,2,5,6-terahydropyridin-2-yl) quinoline and 7-(3-butenyl)-2-hydroxy-6-(1,2,5,6-tetrahydropyridin-2-yl)-7,8-dihydroquinoline and their isomers. Thus, in this study, proposed structures of antioxidant compounds in shrimp are given which may lead to better understanding of the antioxidant effect exhibited by shrimp components. Therefore, successful isolation and structural elucidation of the antioxidant compounds in shrimp, for the first time, is a major contribution to the existing knowledge of antioxidants from natural sources. Production of novel protein dispersions and hydrolysates and evaluation of their properties is another achievement of this research.

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Ahmad, M.M., Al-Hakim, S. and Shehata, A.A.Y. (1983). The antioxidant activity of amino acids in two vegetable oils. *J. Am. Oil Chem. Soc.*, **60**: 837-840.

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APPENDIX

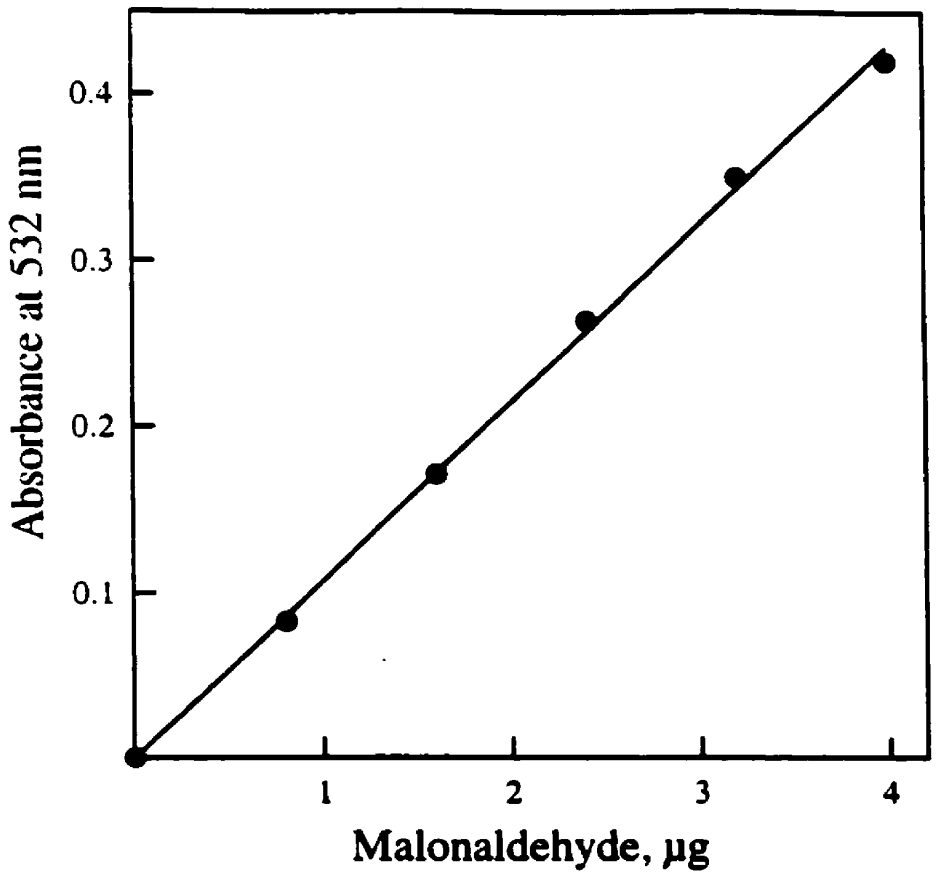

Figure A.1 Standard line of concentration dependence of TBARS as reflected in the absorbance of the TBA-malonaldehyde complex

Regression coefficient (r) = 0.997
Equation of the line was Y = aX+b where,

Y = absorbance at 532 nm
X = concentration of malonaldehyde in 5 mL distillate, µg (C)
a = 0.116
b = 0.0

$A_{532} = 0.116*C$
Therefore, $C = 8.42A_{532}$
Since D grams of sample was used for distilling 50 mL, the TBARS value $= 8.42A_{532} \times 10/D$ (mg malonaldehyde equivalents/kg sample).

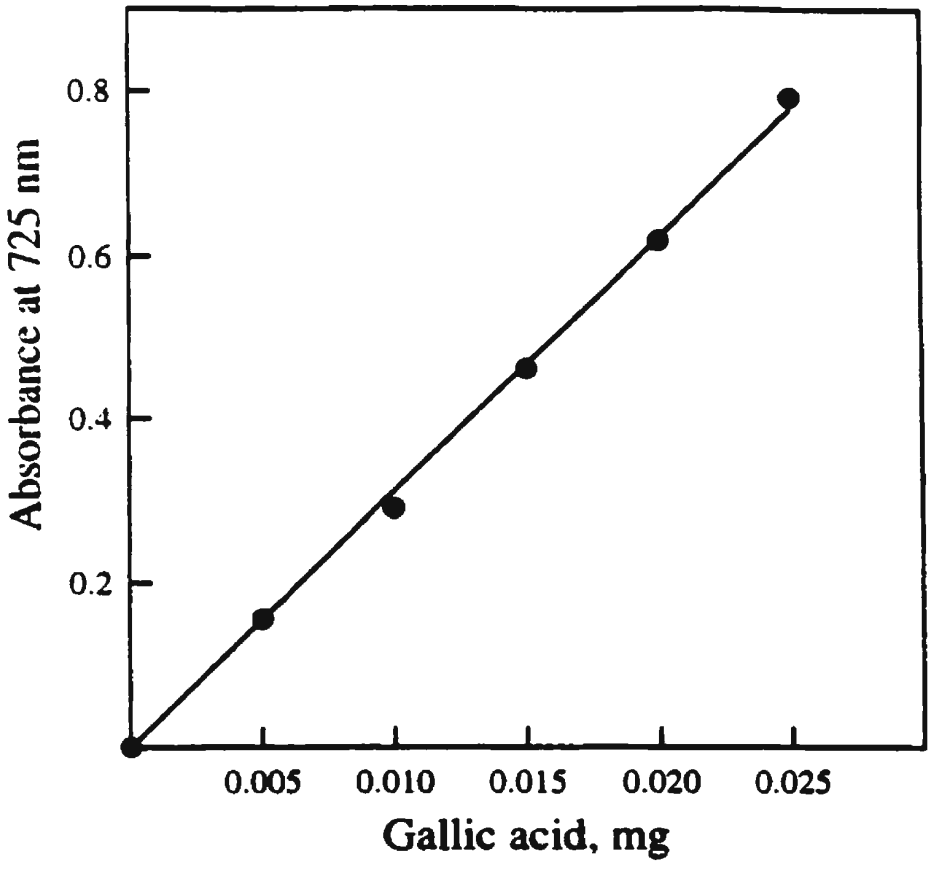

Figure A.2 Standard line of concentration dependence of total phenolics as reflected in the absorbance of gallic acid

Regression coefficient (r) = 0.996
Equation of the line was Y = aX+b where,

Y = absorbance at 725 nm
X = concentration of gallic acid in solution, mg (C)
a = 31.055
b = 0.0

$Abs_{725} = 31.055*C$
Therefore, $C = 0.032A_{725}$
Since shrimp extract have to be diluted, then:
C (mg) = $F(0.032A_{725})$
Where F = dilution factor.

Y = Degree of hydrolysis (%)
X1 = Temperature (C)
X2 = pH
X3 = Enzyme/substrate concentration (AU/kg protein)

Coding Coefficients for the Independent Variables

Factor	Subtracted off	Divided by
X1	55.000000	10.000000
X2	8.000000	1.000000
X3	25.000000	10.000000

Response Surface for Variable Y

Response Mean	16.283333
Root MSE	0.530742
R-Square	0.9543
Coef. of Variation	3.2594

Regression	Degrees of Freedom	Type I Sum of Squares	R-Square	F-Ratio	Prob > F
Linear	3	21.204000	0.4299	25.092	0.0002
Quadratic	3	24.437500	0.4954	28.918	0.0001
Crossproduct	3	1.430000	0.0290	1.692	0.2452
Total Regress	9	47.071500	0.9543	18.587	0.0002

Residual	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	Prob > F
Lack of Fit	5	2.006000	0.401200	4.863	0.1116
Pure Error	3	0.247500	0.082500		
Total Error	8	2.253500	0.281688		

Parameter	Degrees of Freedom	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|
INTERCEPT	1	-88.876250	20.666839	-4.300	0.0026
X1	1	0.634750	0.388335	1.635	0.1408
X2	1	17.917500	5.284518	3.391	0.0095
X3	1	1.029250	0.243838	4.221	0.0029
X1*X1	1	-0.005250	0.003224	-1.628	0.1421
X2*X1	1	0.015000	0.018765	0.799	0.4472
X2*X2	1	-1.125000	0.322422	-3.489	0.0082
X3*X1	1	-0.003250	0.001878	-1.732	0.1215
X3*X2	1	-0.022500	0.018765	-1.199	0.2648
X3*X3	1	-0.011250	0.003224	-3.489	0.0082

Parameter	Parameter Estimate from Coded Data
INTERCEPT	17.825000
X1	0.960000
X2	0.180000
X3	1.080000
X1*X1	-0.525000
X2*X1	0.150000
X2*X2	-1.125000
X3*X1	-0.325000
X3*X2	-0.225000
X3*X3	-1.125000

Factor	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	Prob > F
X1	4	10.987865	2.746964	9.752	0.0036
X2	4	4.338435	1.084609	3.850	0.0496
X3	4	16.343435	4.085859	14.505	0.0010

Canonical Analysis of Response Surface
(based on coded data)

Factor	Critical Value Coded	Uncoded	
X1	0.819650	63.196502	TEMPERATURE
X2	0.099478	8.099478	pH
X3	0.351658	28.516583	ENZ/SUB CONC

Predicted value at stationary point 18.417281

Eigenvalues	Eigenvectors X1	X2	X3
-0.468208	0.952741	0.153680	-0.262045
-1.062880	0.285865	-0.745425	0.602181
-1.243912	0.102792	0.648632	0.754129

Stationary point is a maximum.

Estimated Ridge of Maximum Response for Variable Y

Coded Radius	Estimated Response	Standard Error	Uncoded Factor Values X1	X2	X3
0.0	17.825000	0.208793	55.000000	8.000000	25.000000
0.1	17.960427	0.208674	55.690786	8.011788	25.713386
0.2	18.076034	0.208436	56.447480	8.022880	26.361405
0.3	18.172763	0.208459	57.271792	8.032994	26.931346
0.4	18.251679	0.209501	58.160773	8.043178	27.413110
0.5	18.313914	0.212869	59.106615	8.053534	27.801629
0.6	18.360600	0.220511	60.097887	8.064300	28.098081
0.7	18.392793	0.234792	61.121902	8.075593	28.309214
0.8	18.411424	0.257918	62.166973	8.087438	28.445282
0.9	18.417277	0.291312	63.223714	8.099801	28.517741
1.0	18.410991	0.335417	64.285326	8.112620	28.537568